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EXTRACTION AND HPLC ANALYSIS OF POTATO SPROUT SUPPRESSANT CHEMICALS

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**Thesis submitted for the degree of Doctor of Philosophy
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Dedication

To my mother Khawir Hawwas who passed away during this study; I know how important it was to be with you during your last moments but I hope you accept this humble effort as an apology, I am sure it will make you happy and proud. Thank you for giving me all the love, confidence, support and opportunities to achieve all that I wish. You were a great mother.

Nidhal

Glasgow/2012

Abstract

In the UK, up to six million tonnes of potatoes are produced annually and more than half of this production is stored for the fresh market and food processing. To maintain potato quality from sprouting, chlorpropham (CIPC) is currently used as the main sprout suppressant in commercial potato stores. Questions have been raised about the safety of application of this compound in potato stores due to increasing concern about the toxicity of its residue and degradation products mainly 3-chloroaniline (3-CA) on the potato tubers. To date, there is no realistic replacement of CIPC for inhibiting sprouting of potatoes destined for processing. Searching for alternatives is crucial particularly as most supermarkets demand foods free of chemicals. The sprout inhibitor 1,4-dimethylnaphthalene (1,4-DMN) can be a suitable replacement for CIPC as it is naturally occurring in the potato and currently used in many countries in the world. To introduce this compound to the UK for commercial use, many investigations must be conducted to ensure its safety for human health and the environment. This study intended to focus on the determination of the residue level of 1,4-DMN, CIPC and its metabolite 3-CA in potato and water samples, hence developing analytical methods was required as a preliminary step.

In this study, three HPLC systems were used for validating a separation method for the analysis of 1,4-dimethylnaphthalene and its internal standard 2-methylnaphthalene (2-MeN). Under the same chromatographic conditions, all these systems achieved excellent separation on a Jones-ODS column (Hypersil ODS 5 μm , 250 mm x 4.6 mm) at ambient temperature isocratically using 70% acetonitrile as mobile phase at a flow rate of 1.5 mL/min, 20 μL injection volume, a run time of 10 minutes and a detection wavelength of 228 nm. All three systems showed high precision ($\text{RSD}\% < 1\%$), good linearity of the calibration curves at two concentration ranges (0.02 – 0.1 and 0.2 – 1.0 $\mu\text{g/mL}$) of each 1,4-DMN and 2-MeN with coefficient of determination (R^2) of the regression line of 0.990 or more. The best system SpectraSERIES UV100-autosampler system was selected for the remainder of this research as it offered lower values for both the limit of detection (LOD) (0.001 – 0.004 $\mu\text{g/mL}$) and the limit of quantification (LOQ) (0.002 – 0.013 $\mu\text{g/mL}$) for both compounds.

A second isocratic reversed phase HPLC-UV method was developed and validated for analysis of 1,4-DMN and 2-MeN using methanol as a substitute solvent for standards and mobile phase preparations to overcome the problem of a global shortage of acetonitrile

during 2008 – 2009. The best separation was achieved on the Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Spherclone) column using 90% methanol as mobile phase at a flow rate of 1.5 mL/min and a 6 minute run time. The method was validated producing good precision, linearity and low values of LOQ (\sim 0.001 μ g/mL).

A straightforward and rapid isocratic HPLC-UV method was developed and validated for the simultaneous analysis of both CIPC and its degradation product 3-CA using methanol as a solvent and prothion (IPC) as an internal standard. To achieve high resolution of the three compounds, the chromatographic conditions selected were: Phenomenex[®] column (ODS-2 250 mm x 4.60 mm 5 μ m Spherclone), 62% methanol at a flow rate of 1.5 mL/min, detection wavelength of 210 nm and a 15 minute run time. Method validation confirmed good precision, acceptable linearity and low values of LOD (\sim 0.01 μ g/mL) and LOQ (\sim 0.04 μ g/mL) for CIPC and 3-CA. These proposed HPLC methods are suitable to apply for the determination of the studied compounds in both potatoes and water samples.

Quantitative laboratory analysis of 1,4-DMN, 2-MeN, CIPC and 3-CA in water solutions showed acceptable standard preparations in water with good precision and linearity and lower values of LOD and LOQ close to those obtained in organic solvent preparations. An adsorption study of 1,4-DMN and 2-MeN on laboratory ware showed that glass materials were acceptable to use whereas there was a considerable adsorption to plastic containers and filters. In contrast, 3-CA showed no adsorption onto any of the laboratory ware tested. CIPC also showed good recoveries with most of the materials tested.

In reviewing the literature, no suitable published method for the simultaneous determination of CIPC and its metabolite 3-CA in potato peel was found. A simple analytical method was developed based on methanol-soaking overnight extraction coupled with HPLC-UV for analysis of CIPC and 3-CA in potato samples using IPC as internal standard. The method was validated and the calculated limit of quantification was 0.01, 0.05 and 0.02 μ g/g in whole tuber for CIPC, IPC and 3-CA respectively. The efficiency of the method reported recovery values of up to 90% for both CIPC and IPC through spiking organic potato peel at three spiking levels of 0.8, 8.0 and 80 μ g/g. By contrast, 3-CA recoveries offered very low values of 10 and 23% at concentration levels of 8.0 and 80 μ g/g respectively and no peak was detected at the lower level of 0.8 μ g/g. This method was compared with the routine Soxhlet-GC method used for the analysis of the residues of CIPC in potato samples at the University of Glasgow laboratory and gave results approximately 23% higher residues of CIPC. This new method at this stage was suitable to

extract CIPC in 20 potato samples daily. Nevertheless, an interesting finding was that despite the low recovery of 3-CA it was identified in treated potato samples. This unanticipated low recovery is noteworthy and indicates that the actual residue may be much higher.

A comprehensive study was made to improve the extractability of 3-CA from potato samples investigating parameters including potato variety, extracting solvent, extraction method, spiking procedure and different treatments for potato samples. All these experimental trials showed no recovery improvements, thus four possible mechanisms were suggested for poor recovery of 3-CA including volatilisation, reaction with potato components, enzymatic activity and ion exchange related to pH.

Under the laboratory work experimental conditions, no measurable loss of 3-CA by volatilisation was found. No reaction of 3-CA was found to occur with other potato components under the experimental conditions used. However, the Schiff base reaction and/or hydrogen bonding may link the amino group of 3-CA and some functional groups abundant in potatoes (e.g. carbonyl, quinone). This study also showed a potential role for enzymatic activity in the poor recovery of 3-CA. Using antioxidants or acidity to inhibit this enzymatic activity was shown to enhance the extractability of 3-CA. Binding of 3-CA to potato peel substrates by ion exchange is unlikely as the pK_a value of 3-CA is lower than the pH of the potato. However, using sulphuric acid combined with methanol as an extracting solution improved the recovery. Optimising the extraction process showed that using a mixture of 1 M H_2SO_4 in 50% methanol as an extracting solution for 24 hours at 50 °C improved the extraction recovery of 3-CA up to 85%. This final extraction method was applied for the determination of the residues of both CIPC and 3-CA in commercial potato samples which had received many applications of CIPC, thus reporting high residue values. Additionally, potato samples were taken from different UK stores for the storage season 2010 – 2011 which had received CIPC application at high and low temperature (450 °C and 270 °C respectively) fogging. Analysis of these potato samples showed no significant difference between high and low temperature for the first application of CIPC for both residues of 3-CA and CIPC. A significant increase in both compounds was found between the first and second application at 270 °C indicating a possible build up with time during storage.

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Authors' Declaration

I declare that all the work presented in this thesis is entirely my own work. It has not been submitted for any other professional degree.

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Nidhal Meena Sher Mohammed

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List of Abbreviations

~	Approximately
µg	Microgram
1,4-DMN	1,4-Dimethynaphthalene
2-MeN	2-Methylnaphthalene
3-CA	3-Chloroaniline
ACN	Acetonitrile
ASE	Accelerated solvent extraction
AU	Absorbance unit
CIPC	Chlorpropham
CRMs	Certified reference materials
DAD	Diode array detector
DW	Dry weight
EPA	Environmental protection agency
FAO	Food and agriculture organisation
FID	Flame ionization detector
GC/MS	Gas chromatography/mass spectrometry
GLC-ECD	Gas liquid chromatography-electron capture detection
GLC-NPD	Gas liquid chromatographic-nitrogen phosphorus detection
i.d.	Internal diameter
ICH	International conference on harmonisation
IPC	Propham
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M	Mean
MAE	Microwave-assisted extraction
MeOH	Methanol
min	Minute
mL	Milliliter
mm	Millimeter
MRLs	Maximum residue levels
n	Number of replicates

nm	Nanometer
o.d.	Outside diameter
ODS	Octadecylsilane
PAHs	Polycyclic aromatic hydrocarbons
PLE	Pressurised liquid extraction
PPO	Polyphenol oxidase enzyme
psi	Pound per square inch
PTFE	Polytetrafluoroethylene
r	Correlation coefficient
R^2 (r^2)	Coefficient of determination
RP	Reversed phase
R_s	Resolution
RSD%	Relative standard deviation percentage
S	Slope
S_B	Standard deviation of the blank signal
SD	Standard deviation
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
SPME	Solid Phase Micro-Extraction
TMP	Trimethyl pentane
UV	Ultraviolet absorption spectroscopy
Y_B	Blank signal
λ_{\max}	Maximum wavelength

Chapter 1: General Introduction

1.1 Potato production

Food requirement is increasingly becoming a major source of concern to the people of the world. Potatoes are one of the major food crops for human consumption, either fresh or processed. They are ranked globally as the fourth staple food after wheat, rice and corn in terms of agricultural area, high yield and adaptability to a wide range of climatic conditions and soil varieties (Ghazavi and Houshmand, 2010; Topcu et al., 2010; Burlingame et al., 2009). Potatoes were first domesticated about 8000 years ago in South America and taken to Europe and the UK through Spanish conquerors in the sixteenth century (Lutaladio and Castaidi, 2009).

Over time, globally the potato has been undergoing major changes and increased production. According to the Food and Agriculture Organisation (FAO) statistics, the four biggest potato producing countries in the world rankings in 2009 were China, India, the Russian Federation and Ukraine which produced approximately half of the total world production 330 million tonnes (FAO, 2011). Potato production in the developed countries is more dominant than developing countries, which require improvements in potato variety and disease management. However, potato consumption is expanding gradually in developing countries because potatoes are a vital source of food, employment and income. Additionally, potatoes can be an alternative for costly cereal crops because they are not a globally traded commodity (Lutaladio and Castaidi, 2009). Potatoes are a food energy supply of carbohydrate and protein and in addition they are a rich source of some vitamins including C, B₆, thiamine, riboflavin and niacin. Moreover, they contain appreciable levels of minerals e.g. phosphorus, magnesium, iron, calcium, potassium and various antioxidants (Burlingame et al., 2009; Lutaladio and Castaidi, 2009). The actual amount used for human consumption is 60% of the total production with the remainder going to a range of various other uses such as animal feed, seed tubers, industry and pharmaceutical products (Topcu et al., 2010; Lisinska and Leszczynski, 1989; Sonnewald, 2001).

Potatoes are a seasonal crop and best cultivated in a moderate climate, their varieties are categorised according to their season, planting and harvesting. In the UK, potatoes are planted most often in April and tend to be harvested in September. Most soils are suitable for growing potatoes and they often need moist and slightly acidic soil. However, high acidity of soil may result in small tubers. Potato harvesting depends on the variety and the

area grown. Commercially, in the UK there are approximately six million tonnes produced each year, most intensively in the east of England, the west midlands and south east of Scotland (Cunnington, 2008; Sonnewald, 2001).

1.2 Potato sprouting

After harvest, most of the potato tubers are stored for a short or longer time until being used or distributed to the markets. In reality, the storage period is sometimes longer than these potatoes spend in the ground. In the UK, the total consumption of potatoes including imports is up to 5.5 million tonnes annually. Around 4 million tonnes are stored, approximately 2.5 million tonnes go to the fresh food market while the rest are processed (e.g. crisps and chips). The types of storage are different between fresh sector and potatoes for processing (Cunnington, 2008).

To store potatoes to the correct quality specification, specific storage conditions are required i.e. control of humidity, ventilation, dark places and temperature, to maintain good quality of the potato tuber cultivars. In commercial stores, potatoes should be stored at between 90 – 95% relative humidity to prevent them drying out; in addition, ventilation conditions are required to avoid anaerobic respiration and fermentation. Dark places are important to control the formation of green skin and sprouting.

Potatoes for processing purposes have to be stored at a relatively high storage temperature, usually between 8 and 10 °C. High temperature promotes sprout development, tuber dehydration and shrinkage. Whilst low storage temperatures (2 – 4 °C) can delay sprouting they can also produce potato tubers with a high accumulation of reducing sugars (glucose and fructose), thus changing the potato taste and colour during frying (Pranaitiene et al., 2008; Teper-Bamnolker et al., 2010; Kyriacou et al., 2009).

Sprouting of potatoes is a serious problem causing losses in stored potatoes, it is associated with undesirable changes including weight loss, loss of nutrient value, softening, a high susceptibility to bruising and enzymatic discolouration and increased levels of naturally-occurring toxicants, e.g. glycoalkaloids (Lu et al., 2012; Teper-Bamnolker et al., 2010; Mondy et al., 1993; Mondy et al., 1992b). Most of these changes are perhaps due to the evaporation and transport of nutrients as energy into the sprouts.

Breaking the dormancy period (particularly endodormancy) in the potato tubers begins sprouting. Dormancy can be defined as a complex set of physiological states and

conditions in which plants respond to a series of stresses such as drought and overwintering by entering a state of growth suspension (Campbell et al., 2008; Teper-Bamnolker et al., 2010). In general, the dormancy period is a period when no bud growth can take place. While endodormancy is specifically defined as the dormancy period when sprouting can be controlled under genetic and environmental factors during growth and storage such as temperature, irrigation and light exposure (Teper-Bamnolker et al., 2010; Sonnewald, 2001). Generally, potato sprouts occur in long term storage in particular during winter storage. Therefore, extending the dormancy period during storage is necessary to control and prevent early sprouting.

1.2.1 Methods for sprout control

Sprout control of potatoes tubers is an important issue for potato storage to maintain the desired quality of harvested potatoes. Sprouting of potatoes during storage can be suppressed by several approaches including use of long dormancy cultivars, controlling of some factors in the stores (e.g. low storage temperature, light and irradiation) or employing chemical treatment (Daniels-Lake et al., 2011; Kraish, 1990; Mondy et al., 1992b). Brief details of the approaches for sprout control are summarised below:

Storage at low temperature can delay sprouting but there is the possibility to convert the starch into reducing sugars due to disequilibrium between starch turnover and the glycolysis rate (Sonnewald, 2001). This can cause undesirable changes in taste with increasing sweetness of the tuber. In addition, a browning can occur due to Maillard reactions that take place when tubers are processed into French fries and chips at high temperature (Saraiva and Rodrigues, 2011). Therefore, processing varieties require a high quality of storage performance. Desirable properties of processing potato varieties are long natural dormancy under sprout promoting temperature ($> 5\text{ }^{\circ}\text{C}$) and low reducing sugar accumulation under low storage temperature conditions ($< 10\text{ }^{\circ}\text{C}$) (Kyriacou et al., 2009). Storing potatoes at low temperatures is costly and only suitable for fresh pack and organic production systems.

Breeding is a natural method to control sprouting through developing varieties with longer dormancy. However, it is a long procedure and preserving the quality characteristics is required (Singh and Kaur, 2009).

Light has an effect on sprouting. It does not influence the dormancy period, the effect is only on the growth of the sprouts when the dormancy is broken (McGee et al., 1987). It

was suggested that storing in the dark at 5 – 8 °C could prevent sprouting for a long period of time (6 months) (Sengul et al., 2004). In addition, a dark place is essential to avoid developing a green skin on the potatoes which occurs due to chlorophyll accumulation and the formation of glycoalkaloids; these have a toxic effect on humans (Sengul et al., 2004; Haase, 2010). For this reason, it is recommended to cut the green skin off potatoes before consumption.

Irradiation has been recognised to be a means of sprout inhibition since the 1950s in numerous countries. The use of gamma rays or low energy electrons can effectively inhibit sprouting of potato tubers through penetration of these energies into the surface of the tuber where the eyes of the potato are located. Advantages of this treatment are long-term suppression of sprouting and little chemical residue thereby promoting potato safety. However, using irradiation to inhibit sprouting is very limited in the potato industry, it necessitates many facilities and is costly, which restricts the use of the technology. In addition, it is known to affect the molecular size of potato starch leading to degradation of the polysaccharide chains. Many countries restrict using this method on food (Lu et al., 2012; Kleinkopf et al., 2003; Todoriki and Hayashi, 2004; Saraiva and Rodrigues, 2011; Kumar et al., 2009).

Hot water dip and vapour heat treatment at temperatures ranging from between 50 to 80 °C for various durations can be used for sprout suppression of potatoes when applied at the storage emergence stage. However, longer duration has undesirable side effects causing discolouration of the skin (Rama and Narasimham, 1986).

Controlled atmosphere (CA) storage is a combination of high CO₂ and low O₂ and can be used to control sprouting during storage. Some of the disadvantages of this method are that it requires an airtight room that is costly and causes dark coloured fries and perhaps increased tuber disorder and diseases (Singh and Kaur, 2009; Khanbari and Thompson, 1994). Moreover, controlled atmosphere storage at a low level of O₂ below the respiration requirement can be responsible for increasing sugar that causes the formation of acrylamide during potato frying, acrylamide is a known human carcinogen (Gokmen et al., 2007).

Pressure processing is a new technique under experimental study to control potato sprouting including the use of pressure treatments as a non-thermal and environmental friendly method which is chemical-free. This method is increasingly interesting for food

processing and preservation methods. However, before using in potato stores and being commercially available for processing, more investigations are required regarding the physiological and metabolic processes of the inhibitory effect on potato sprouting (Saraiva and Rodrigues, 2011; Oey et al., 2008).

Chemical suppressants are applied to the potato tubers during the storage period. The tubers show significantly less tuber sprouting than untreated tubers. Generally, treated potatoes have smaller weight losses compared with untreated potatoes (Pranaitiene et al., 2008).

1.2.2 Chemical sprout inhibitors

Using chemical sprout suppressants in combination with appropriate storage management is the most effective way for successful long term storage and to inhibit sprouting of potato tubers, without reducing the storage temperature in commercial stores. Ideally, a potato sprout suppressant should have several properties (Teper-Bamnolker et al., 2010; Vaughn and Spencer, 1991; Beveridge et al., 1981a) that can be summarised below:

- Effectively inhibit sprouting under commercial storage conditions and at low dosage rates.
- Suitable for use on potato tubers and have minimum effect on their quality (i.e. weight loss, sugar content, appearance).
- Low toxicity and its residues do not cause problems to humans.
- Rapidly broken down and environmentally friendly.

A range of sprout suppressing chemicals will be discussed in this study. Some are commercially available, others require more investigation to be used in potato stores and some others have been banned.

1.2.2.1 Commercially used

Chlorpropham

Isopropyl-N (3-chlorophenyl carbamate) commonly known as CIPC, has been used traditionally and is the most effective sprout suppressant registered and currently used. CIPC has been used for about half a century in commercial potato storage in numerous

countries in the world (Rentzsch et al., 2012; Lu et al., 2012; Verhagen et al., 2011; Daniels-Lake et al., 2011; Teper-Bamnolker et al., 2010; Boylston et al., 2001).

1,4-Dimethylnaphthalene (1,4-DMN)

It was discovered as a new sprout inhibitor in the late 1970s and known as the trade name DMN. It is available commercially, mainly as 1,4SIGHT in the USA, Canada and New Zealand but not in UK to date. Other isomers of naphthalene have also been shown to have good sprout suppressant properties and maintain the quality of treated tubers (e.g. 1,6-DMN, 2,3-DMN and 2,6-diisopropylnaphthalene (2,6-DIPN)) (De Weerd et al., 2010; Beveridge, 1979; Lewis et al., 1997).

Propham

Isopropylphenylcarbamate or IPC is a herbicide from the same class as chlorpropham. It was applied commercially to prevent sprouting, mostly in combination with chlorpropham, but currently its application has been banned in most countries (not supported in the EU) due to ecological concern.

Maleic hydrazide

1,2-Dihydropyridazine-3,6-dione or MH is an old well known synthetic plant growth regulator and sprouting inhibitor (Mamani Moreno et al., 2012) which is widely used on storage potatoes in the USA, Europe and Canada. It is used commercially as a formulation of the potassium salt and does not affect the crop yield and quality, and does not produce phytotoxicity symptoms in the foliage (Caldiz et al., 2001). Application of maleic hydrazide differs from other sprout inhibitors, as it is applied in the field and penetrates the leaf and is translocated into the tuber flesh. The time of spraying is delicate and must be performed before defoliation. The timing is critical and unfavourable in the UK due to poor weather conditions which reduce the uptake of the chemical by the leaf cuticle, resulting in reduced tuber size (Duncan et al., 1992). It is employed to control potato volunteers which are left in the field after harvest.

Carvone

A common monoterpene chemical extracted from caraway seed, it has been shown to be an efficient potato suppressant and decreases the rate of microbial activity on the potato tuber. It is commercially used as a sprout inhibitor in several countries, for example Holland and Switzerland; however, the mechanism of sprout inhibition is not completely clear. Due to being a natural product derived from plants, carvone can be commercially exploited in

organic potato stores and is expected to leave little or no residue. However, it is costly and can affect the potato taste (Rentzsch et al., 2012; Sanli et al., 2010; Oosterhaven et al., 1995; Teper-Bamnlker et al., 2010; Kleinkopf et al., 2003; Hartmans et al., 1995). Currently, there is no use of carvone in the UK.

Ethylene

Ethylene gas is effective at suppressing potato sprouting, however it affects sugar metabolism and often produces undesirable fry colour (darkening) when used alone (Daniels-Lake et al., 2011). It was postulated that the activity of sprout inhibition of ethylene depends on the concentration and the duration of exposure. Ethylene was registered for use in Canadian stores in 2002 as a sprout controlling agent during long term storage of potatoes. In the UK, it was launched to be used at low temperatures in commercial stores according to conditional approval in 2003 to become an acceptable replacement for chlorpropham. The application cost is cheaper than using chlorpropham, in addition it is safer to humans. Its use for the fresh potato market was a major step forward; however, more study is required to understand the principle effect of ethylene for possible use for the processing market, in particular, quality issues, such as sugars and texture need more investigation (Prange et al., 2005; Daniels-Lake et al., 2005).

H₂O₂

Hydrogen peroxide is being evaluated to control tuber dormancy and sprouting by physically damaging the growing sprouts or buds before they can extend. Applying a high dose and several applications of hydrogen peroxide will be sufficient to prolong the dormancy period and inhibit sprouting. Another advantage of hydrogen peroxide is that it has some antimicrobial activity which is beneficial if used in potato stores (Kleinkopf et al., 2003; Bajji et al., 2007). Hydrogen peroxide is applied to control sprouting in organic potatoes in some countries where its use is permitted.

Tecnazene

Initially tecnazene was used as fungicide and to control sprouting of potato seeds when applied as a dust or granular formulation. It has been used in commercial potato stores in the UK for over 40 years but there is concern about its residue in soil and water. In addition, it's unacceptably high toxicity contributed to it being banned 10 years ago (not supported in the EU).

1.2.2.2 Other sprout inhibitors

Essential oils (e.g. caraway, peppermint, spearmint, clove oil and Smart Block) extracted from plant materials have been used as environmentally friendly sprout suppressant alternatives to CIPC that can inhibit early sprouting of potato tubers. Many experiments illustrate the potential of thermal fogging with mint essential oil as a potato sprout inhibitor that has a negligible environmental impact. However, its high volatility can be one of the disadvantages for application in potato stores (Rentzsch et al., 2012; Kleinkopf et al., 2003; Teper-Bamnolker et al., 2010). It was recommended to use a suspension of dill and caraway seed oil using 22 – 25 g/tonne of oil and 2.2 – 2.5 L/tonne of water as natural inhibitors to treat organic farming potatoes because they leave behind little or no residue (Pranaitiene et al., 2008).

Volatile monoterpenes are natural constituents which are easily obtained from essential oils like 1,8-cineole and can suppress sprouting. 1,8-cineole may be used as a potential alternative to currently used synthetic potato inhibitors as it possesses several benefits; its high volatility at cool store temperature, weak toxicity, widely available (eucalyptus oil 85 – 95% v/v) and low cost (Vaughn and Spencer, 1991).

Ozone is a powerful oxidiser that is damaging to living tissue. It can also prevent potato sprouting but is not commercially used, although it is supposed to be another alternative for CIPC because of its rapid decomposition and low cost (DanielsLake et al., 1996).

Aromatic aldehydes and aromatic alcohols. Several natural volatile compounds possessing low toxicity were identified which inhibited sprouting when applied to potato tubers as volatiles or directly as emulsions such as salicylaldehyde, benzaldehyde and cinnamaldehyde. They were shown also to inhibit the growth of fungi and maintain the potato tubers firmness (Vaughn and Spencer, 1993).

1.2.3 Sprout inhibitors of interest in this study

In this study, the focus will be on chlorpropham (CIPC) and 1,4-dimethylnaphthalene (1,4-DMN) because they are effective chemicals available commercially for sprout control and quality management. Additionally, increasing concerns have been raised recently regarding the toxicity of CIPC, acceptable residue level on potatoes and safety of CIPC application in potato stores (Campbell et al., 2010; Teper-Bamnolker et al., 2010; Boylston et al., 2001). This was confirmed by whole diet studies by the USA food and drug administration (FDA) which indicated that CIPC is considered to be one of the most abundant pesticides in the

diet of adults. In addition, it comprises 90% of the pesticide residues found in potatoes (Daniels-Lake et al., 2011). Therefore, several alternatives have been suggested for the replacement of CIPC (Kleinkopf et al., 2003; Prange et al., 2005), including 1,4-DMN which has been used in the USA since 1996 and also in some other countries (De Weerd et al., 2010).

In the UK, CIPC is the main pesticide used as a sprout suppressant. Due to the concern over CIPC, 1,4-DMN could be an alternative to CIPC. It can be applied commercially either alone or as a supplement to CIPC (Campbell et al., 2010; Kleinkopf et al., 2003). 1,4-DMN is particularly effective in achieving optimal results in stopping sprouts on potatoes previously treated with CIPC when compared with CIPC alone.

1,4-DMN is a naturally volatile chemical produced in potato tissues and other plants and therefore there are fewer toxicity issues (Meigh et al., 1973; Beveridge et al., 1981a). MacLeod et al. (2004) reported that there was no evidence that 1,4-DMN is naturally occurring in potatoes and they thought that this polycyclic aromatic hydrocarbon (PAH) can be absorbed by plants from root and aerial exposure (MacLeod et al., 2004). But, it was confirmed that approximately 50 ng/kg of 1,4-DMN was found to be present naturally in potato (Harry Duncan, personal communication). In reviewing the literature, it is clear that 1,4-DMN is not a reproductive toxicant.

The potential of 1,4-DMN as a sprout suppressant of 1,4-DMN has been reported by Beveridge et al. (1981a). However, it was found that a single application of 1,4-DMN was insufficient for long term storage of potatoes tubers and further applications were required to control sprouting (Beveridge et al., 1981b). Several studies have been carried out comparing the efficiency of the sprout suppressants 1,4-DMN and CIPC. Lewis and co-workers (1997) showed that two thermal fog applications of DMN at rates of 200 and 300 mg/kg were equal to one application of CIPC at rate 22 mg/kg and all treatments of diisopropylnaphthalene (DIPN) at a rate 300 mg/kg in controlling sprouting in Russet Burbank potatoes during ten months of storage. In addition, DMN either applied once or twice resulted in lower residue concentrations on potato tubers than CIPC.

1,4-DMN along with the sprout suppressants carvone and ethylene were compared with CIPC for their efficiency to prevent sprouting, undesirable fry colour and sugar content of potato tubers (Kalt et al., 1999). It was found that all these treatments suppressed sprouting although CIPC offered the best sprout suppression at the end of 25 weeks storage, whereas

the amount of sprouting was greater in the 1,4-DMN treated tubers. DMN was applied in this study at a recommended commercial storage level (20 mg/kg). Higher rate and repeated applications would undoubtedly prevent this sprouting. Fry colour was darker in the ethylene treated tubers than in potatoes treated with other inhibitors and there was no change in the sugar content of tubers treated with DMN.

The efficacy of sprout suppressants including S-carvone and 1,4-DMN was compared to that of CIPC. DMN was found to be as effective as CIPC at 60 mL/tonne whereas S-carvone applied at 600 mL/tonne was similar to that of CIPC applied at commercial rates. These chemicals achieved better sprout control when they were applied before any visible signs of sprouting. The Russet Burbank cultivar was found to be more amenable to sprout inhibition by S-carvone and DMN than the Denali potato cultivar. Unlike CIPC, DMN and S-carvone are fully reversible sprout inhibitors which makes them suitable for application on seed potatoes (Baker et al., 2002).

The mode of action of the sprout suppressants CIPC and 1,4-DMN is distinct. CIPC is a synthetic compound that works externally on the potato surface as a mitotic inhibitor interfering with cell division. More specifically, CIPC can disrupt the spindle formation altering cellular structure and function to inhibit mitosis and prevent sprouting (Vaughn and Spencer, 1991; Kleinkopf et al., 2003). Whilst, the mechanism of action of DMN as a sprout suppressant is still unknown, it is thought to be hormonal in action. 1,4-DMN has proved to be successful for suppressing sprouting as a temporary dormancy enhancer to extend the dormancy period when it penetrates into potato skin. During this physiological state of natural dormancy, DMN inhibits the forming of peeps (< 2 mm sprout tips) and the developing sprout on the potato skin under proper conditions (De Weerd et al., 2010; Kleinkopf et al., 2003; Knowles et al., 2005; Campbell et al., 2010). However, recent research by Campbell et al. (2010) suggested that the role of DMN is not as an enhancing agent of the dormancy state but rather prevents sprouting by some other unknown mechanism. These authors also demonstrated through the DNA microarray profiles that there were significant differences between CIPC and DMN treated meristems, particularly in transcript profiles derived from treatment with either CIPC or DMN and from the dormant state. These results reported the mode of action of the two sprout inhibitors as being different and not due to a prolongation of the normal dormant conditions.

1.2.3.1 Application in commercial stores

1,4-DMN

1,4-DMN is a liquid chemical with a high vapour pressure that provides good volatility (see Figure 4:1). It is applied on potatoes as an aerosol fog using aerosol generating equipment which should be located to supply good distribution and equal amount of spray in the store. DMN is applied at rate of 10 – 20 mL of liquid product per tonne of potatoes. The application allows for a maximum rate of 80 – 120 mL/tonne (4 – 6 applications) over the entire storage season for both fresh market and processed potatoes (John Forsythe, personal communication).

The chemical is injected into a heated chamber (232 – 260 °C) and the vapour generated is blown into the store and re-circulated by an air stream through the potato pile. Storage facilities should be at temperatures between 3 and 8 °C with little outside ventilation. The first application is made after placing the potato tubers into store. Practically, the treated area has to be closed to permit optimum absorption into the tubers at the same time avoiding direct contact of the aerosol fog with the potato tubers. Subsequent re-applications are necessary when visual assessment indicates that the developing bud is sprouting, specifically every 30 days, although the response to inhibitors is different among different potato varieties. Mostly, commercial application is carried as a hot fog, but unlike CIPC cold application can also be performed and has a number of benefits since it does not heat up the store during application and in addition, application can be carried out remotely with a system of timers. However, there are several disadvantages, one is the high vapour pressure required to get good circulation through the pile and an adequate DMN residue on the potatoes, which is difficult to achieve and the second is that cold fog is a mist, rather than a vapour, that tends to condense as liquid droplets on the potatoes. This can lead to direct burning of the skin of the potatoes (John Forsythe and Jim Zalewski, personal communication). In the USA, DMN can be applied consecutively with CIPC and the rate of application is not much varied. The CIPC is applied first (16 – 22 g/tonne one time as a single application) and then the 1,4-DMN is applied on a number of occasions to manage sprouting.

CIPC

In the UK, CIPC is used as the main sprout suppressant used to prolong the storage period and maintain the quality of approximately 90% of ware potatoes destined for processing (Harry Duncan, personal communication). It can inhibit sprout development for between 4

and 8 months. The storage period in the UK starts from September or early October until the end of June the following year.

Generally, the first treatment with CIPC is conducted about two to three weeks after storage when the wound healing (suberisation) period is complete and before dormancy break or initiation. Ideally, this is prior to the eye opening stage and usually in mid to late October or during cold weather. If sprouting starts directly after the first treatment then the rate of CIPC application was not sufficient and should be increased. Subsequent treatments are made when required to manage sprouting. Commonly four treatments per season are required and the interval between two applications is about 6 – 12 weeks, the last application being three weeks before removal from the store. However, a new registration in the UK permits CIPC application two days before store emptying for either processing or for the fresh market (Harry Duncan and Geraldine McGowan, personal communication).

Application and uniform distribution of CIPC in the store requires control of important storage parameters such as temperature, relative humidity, air circulation and quantity of CIPC. Each potato cultivar varies in dormancy period and sprouting mode. The exact concentration of CIPC required to control sprouting will vary. The CIPC application regime and storage conditions depend on whether the raw commodity is destined for the fresh market or processing (frozen, dehydrated product, chips)(Kleinkopf et al., 2003).

The rate of CIPC application differs between the types of store vis bulk or box store. CIPC treatment in box stores is more challenging and the distribution among each individual tuber is more difficult than in bulk store due to the fact that the potatoes are separated in boxes and not well ventilated. However, in a box store, the history of the potatoes in relation to their original farmer and variety is easier to know. In contrast, in a bulk store this is more difficult to predict as the potatoes are mixed from different farms. The maximum amount of CIPC that can be applied to fresh market potatoes should not be more than 36 g/tonne during the storage season, whereas for processing 63.75 g/tonne during the season is the total allowed (Harry Duncan, personal communication).

Application of CIPC is carried out by a thermal fogging process whereby the chemical is introduced in a particulate form to tuber eyes and therefore affects sprout control. To minimise sprouting, the store temperature should be kept at between 5 and 8 °C. Currently, applications in UK stores are made either using a formulation of CIPC (50% w/v) in methanol or dichloromethane as the solvent, or as the solid ingredient alone. Methanol is

preferred more than the chlorinated solvent for both human health and environmental considerations. However, attention should be given to the methanol formulation because it is a flammable solvent (flash point 11 – 12 °C). Thermal fogging of a formulation of CIPC in a carrier solvent of methanol comprises passing the CIPC solution through a fog generator. This aerosol device contains a combustion chamber which can be heated by propane to vapourise CIPC to microscopic crystals which make up the fog, at a burner temperature of 300 – 600 °C and a fuel pressure of ~ 50 psi, although recommendations were made to use lower temperatures (300 – 350 °C) to prevent the possibility of CIPC breakdown at high temperatures (Harry Duncan, personal communication). The fog distributes into the store through a metal aluminum pipe. In the store, circulating air carries the CIPC fog into the air ducts and moves it through the potato pile. An effective distribution depends on many factors such as tuber condition, the store type and design, ventilation and weather conditions during CIPC application. Usually, the application time depends on the size of the store and the potato quantity. Fog application in the UK to treat a store capacity of 1500 – 2000 tonne of potatoes takes about 45 – 60 minutes. The dose of CIPC is varied depending on potato cultivar, store temperature and time of storage (Harry Duncan and Geraldine McGowan, personal communication).

Recently, in the UK, another method of treating potatoes to inhibit sprouting is by melting and forming aerosols from solid CIPC at a rate of 12 – 14 g/tonne. This technique comprises melting solid blocks of CIPC at a temperature of about 37 °C (close to the CIPC melting point) in a heated zone. Hot liquid CIPC is passed into a pump, which carries the molten CIPC to the fog generator to convert the CIPC into a fog by feeding it into a combustion thermal fogger at temperatures usually lower (315 – 340 °C) than with a formulation of CIPC in methanol solution (up to 600 °C). However, the burner temperature is generally the store applicators choice (Harry Duncan and Geraldine McGowan, personal communication). In this manner, a stable fog of CIPC is formed directly into the potato store. To supply a more consistent distribution of CIPC fog in bulk store, slow speed ventilation is required; therefore, fans are connected with a variable frequency drive and run during CIPC application. Also, the fog is re-circulated for more efficient distribution throughout the potato pile and the headspace of the store (see Figure 1:1) (Cunnington, 2008).

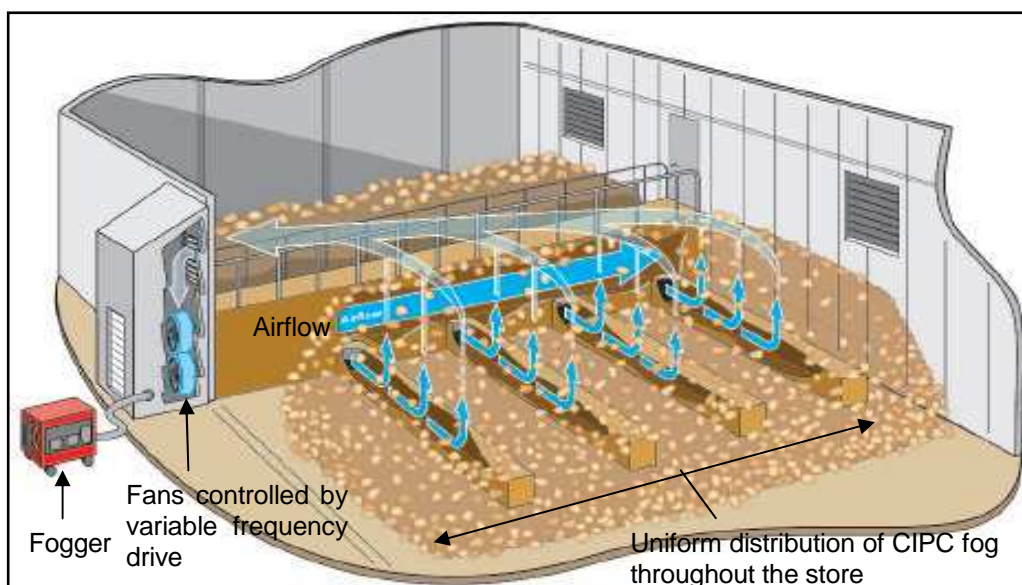


Figure 1:1. Schematic diagram of a UK bulk potato store during application of CIPC (Cunnington, 2008).

1.2.3.2 Factors affecting CIPC application

Occasionally, application of CIPC to control sprouting can fail or be inefficient causing many problems (Kleinkopf et al., 2003; Park, 2004; Park et al., 2009). The reasons for this are summarised as follows:

1. Inappropriate store design and/ or a malfunctioning ventilation system can cause variable temperature from the top to the bottom of the potato pile.
2. Improper sizing, spacing and location of air ducts possibly will result in fluctuating temperature and poor air circulation in the potato pile causing an uneven distribution of the CIPC.
3. Incomplete sealing of the store can result in a significant loss of CIPC through vents.
4. Hot spots from diseases, excess soil and debris in the potato pile and thereby plugging air vents can cause temperature fluctuations and increased respiration of the tuber that encourage sprouting.
5. Potatoes grown under stressed conditions in the field (e.g., water supply, disease, nutrition, temperature) may respond differently to CIPC treatment.

6. Delayed application time after dormancy break produces unsuccessful sprout inhibition.
7. Potato varieties differ in dormancy period and this affects the response from CIPC.
8. Application equipment (e.g. fogger, fans) operate inefficiently.
9. High temperature combined with metallic pipes and long storage period can breakdown CIPC.

1.2.4 Potential breakdown of CIPC to 3-chloroaniline

CIPC is as pesticide of a well known group of N-phenyl carbamates and composed of the ester of carbamic acid. This group is thermolabile and highly sensitive to degradation by fragmentation and/or rearrangement under conditions of inappropriate solvent and excessive heating (Przybylski and Bonnet, 2009; Paiga et al., 2009). CIPC can decompose at 150 °C (Camire et al., 1995) and its degradation products are often active as well.

Application of CIPC by thermal fogging in potato stores can produce large droplets and may be the cause of thermal degradation of CIPC. A recent study of CIPC in the atmosphere of potato stores treated with CIPC showed the presence of another compound, which was later identified as 3-chloroaniline (3-CA) (Park, 2004). The presence of 3-CA in air samples was suggested to be the product of thermal degradation of CIPC during the fogging application. The high temperature of the fogging machine which most often ranges from between 300 – 600 °C and contact of CIPC with hot surfaces (such as the aluminum metallic pipe used to carry the fog into the store) promoted the thermal degradation. The thermostability of CIPC was investigated by Nagayama and Kikugawa (1992) who concluded that CIPC underwent thermal degradation and rapidly changed to produce 3-CA after heating at between 210 °C and 250 °C for 20 minutes. 3-CA and other products of CIPC degradation have also been identified in stored potatoes (Heikes, 1985; Worobey and Sun, 1987).

Additional to thermal degradation, warming the stored potatoes and moisture may encourage bacterial growth, causing microbial degradation of CIPC. Several studies have been reported on the microbial degradation of CIPC which leads to the formation 3-CA (Verhagen et al., 2011; Kaufman and Kearney, 1965; Kearney and Kaufman, 1965). Bacterial degradation of CIPC is suggested as a dominant degradation pathway under

certain environmental conditions, perhaps due to the slight solubility of CIPC in water (see Section 4.3.2.1) and its resistance to the oxidation process (Verhagen et al., 2011; Wolfe et al., 1978; David et al., 1998). The rate constant for the formation of 3-CA was found to be the same as the rate constant for the loss of CIPC, indicating that hydrolysis of the ester bond is the initial step in the microbial degradation of CIPC to form 3-CA, isopropanol and carbon dioxide (Wolfe et al., 1978) as shown in Figure 1:2.

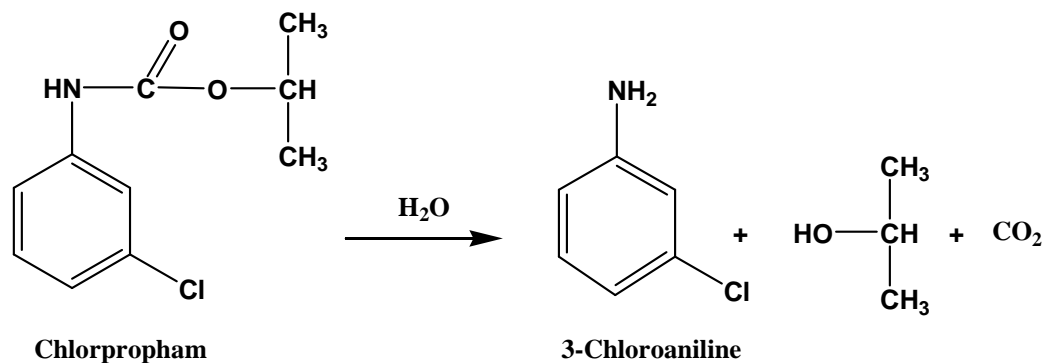


Figure 1:2. Shows the equation of the breakdown of CIPC to yield 3-CA.

Therefore, the existence of 3-CA in potato stores could result from either or both of the two processes: thermal breakdown during CIPC fogging application or microbial degradation that will occur during long storage periods. To examine which process is responsible for the formation of 3-CA, a study was conducted where air samples from potato stores were collected immediately and then 18 hours after thermal fogging application at 600 °C temperature using metal ducting pipe. Samples taken immediately after fogging would mean that no time is allowed for microbial activity. 3-CA was detected in these atmospheric samples and its level was found to be 2 – 3 µg/L immediately after the fogging application, declining to 0.2 µg/L 18 hours after application (Park et al., 2009).

As stated above, there are two processes which account for the presence of 3-CA in the potato store; thermal breakdown and microbial degradation. However, it should be mentioned that based on the lack of moisture present in UK potato stores, the microbial breakdown of CIPC is not suggested as a significant pathway for the formation of 3-CA found in the store atmosphere, but microbial activity in some diseased tubers may promote the potential degradation of CIPC to produce 3-CA (Harry Duncan, personal communication). Additionally, microbial degradation is more likely to occur in water and soil. The presence of 3-CA in potato wash water was investigated by collecting samples of effluents from potato washing plants (Park et al., 2009; Park, 2004). 3-CA was identified either with CIPC or alone in some cases. Although no interpretation was found for this,

microbial activity was suggested as a mechanism to draw a correlation between the formation of 3-CA and disappearance of CIPC.

It was also noted by Park et al. (2009) that 3-CA could be present in the potato store in the CIPC formulation as a minor manufacturing impurity at levels that are strictly controlled (i.e. 0.05% of CIPC by weight).

Taking all the above into consideration, the presence of 3-CA might be a big concern for the potato industry in the following situations (Harry Duncan, personal communication):

1. Store atmospheres immediately after thermal fogging of CIPC application.
2. On the potato tubers.
3. The store fabric.
4. Wash water for potato tubers treated with CIPC and destined for processing or fresh markets.

It should be pointed out that 3-CA levels found in potato stores and potato washing water may be very low and could be missed by routine analysis. However, the concentration of 3-CA can be reduced further by modifying the fogging process of CIPC through lowering the temperature of the application and avoiding metal pipes used to carry CIPC fog into potato store. This control of CIPC application could be used to control thermal degradation but would not reduce levels of 3-CA due to the microbial degradation which is an issue that needs to be resolved.

1.2.5 Health and environmental consideration of studied compounds

1,4-DMN is a compound of a well known group of polycyclic aromatic hydrocarbons (PAHs) and many compounds from this group are recognised to be carcinogenic. This chemical is highly volatile and possesses low solubility in water (see Section 4.2.2.1). It is considered to be toxic to aquatic organisms. However, Health Canada Pest Management Regulatory Agency RD2011-06 (2011) reported that due to its natural occurrence in potato and limited environmental exposure, there are no risks to the public or the environment. Based on the use pattern, no or very limited exposure of aquatic ecosystems is expected. It

is predicted to be degraded rapidly through photochemical reactions and/or microbial activity (Canada, 2011). The registration decision of an application for use of 1,4-DMN in UK commercial potato stores requires more study in order to understand the possible risks to the environment regarding waste peel, water and soil.

Application of CIPC on stored potatoes may lead to risk for humans and the environment in uncontrolled circumstances. According to the cancer classification guidelines of the USA Environmental Protection Agency (EPA/738/F-96/023), there are two risk scenarios for CIPC and its metabolite 3-CA in dietary risk assessment. The first one is based on their residues solely on stored potatoes. While the second scenario was drawn on the term of the “local milk shed” when potato peelings are used to feed cattle and subsequently residues can be present in beef liver and in milk, which will be distributed locally (EPA, 1996). Considering these concerns, available data shows that application of CIPC under proper label directions does not involve undesirable risks to human health or the environment. The toxicological effects and safety data of CIPC are similar to its metabolite 3-CA. For human risk assessment, however, there is a concern over 3-CA which as a derivative of aniline, an aromatic amine that is known to be dangerous to humans and the environment (Sihtmaee et al., 2010). 3-CA is an irritant to eyes and skin if present in sufficient quantities causing redness and swelling of skin and membranes. These are temporary effects which can be resolved shortly after cleaning the skin. Exposure to humans at high levels through air, skin contamination and ingestion can cause different symptoms including dizziness, headache, nausea, vomiting and unconsciousness. Monitoring data indicate that the probable routes of human exposure to 3-chloroaniline are potentially through ingestion of food and drinking water (SRC, 2011). 3-CA is a mammalian metabolite of CIPC. Approximately 20% of CIPC taken into body may be metabolised to 3-CA. 3-CA entering into the human body at high levels causes toxic effects in the blood but these effects are temporary.

The big concern over 3-CA is because it is structurally similar to 4-chloroaniline which is classified as possibly carcinogenic to humans, Group 2B (IARC, 1993; Sihtmaee et al., 2010). However, it should be made clear that 3-CA is not known to be carcinogenic. Additionally, the chemical structure of CIPC is such that only 3-CA, and not 4-chloroaniline, could be produced as a metabolic product.

Due to their solubility in water (see Section 4.3.2.1), CIPC and 3-CA can be present in both soil and surface waters, thus there is the potential for them to be moderately toxicity to aquatic systems. The Environmental Protection Agency provided a list of substances

which are considered to present a potential risk of pollution where they are discharged to, or detected in, groundwater bodies. The agency listed both CIPC and 3-CA as List I and hazardous substances which should be avoided in ground water (EPA, 2010). In addition, according to European Community pollutant Circular No 90 – 55 (1990), 3-CA is recognised to be a toxic water pollutant and harmful to aquatic life (David et al., 1998).

Chloroanilines exist in the environment as degradation products of various pesticides (Rouchaud et al., 1986b). 3-CA has been identified as the main degradation product of the microbial oxidation of CIPC during field soil studies (Rouchaud et al., 1987). 3-Chloroaniline may undergo biodegradation in nature and presence of bacteria cultures and the intermediate metabolite is 4-chlorocatechol (Kondo et al., 1988; Paris and Wolfe, 1987; Reber et al., 1979). Ultrasonic and photochemical degradation of CIPC in aqueous solution have been studied (David et al., 1998). 3-CA was identified as the main ultrasonic degradation product by HPLC and by GC-MS. Photolysis of chlorpropham did not form 3-CA directly but it was observed in its biotransformation.

Taking all the above considerations into account, the level of CIPC in stored potatoes needs to be monitored to reduce any risks for human health and the environment. An important consideration should be given to the presence of CIPC and its metabolite 3-CA in potato wash water due to their toxicity. Due to the requirement to recycle water, water issues are very high profile at present and wash water needs to be cleaned up before release, otherwise there is a danger of releasing both CIPC and 3-CA into the environment (Harry Duncan, personal communication).

1.3 Sprout inhibitor residues in potatoes and factors influencing their presence

A consequence of the application of potato sprout inhibitors with different formulations (i.e. gas, fog, aerosol, dust powder, granular and emulsifiable concentrate) throughout the storage season may be that some residues remain on the potato tubers and such residues may subsequently be transferred to other environmental samples such as water and soil. Thus, the residue levels of these compounds in potato tubers have become increasingly subject to regulation and are a major concern for consumer safety.

1,4-DMN

Nowadays, the commercial use of 1,4-DMN as a naturally occurring potato sprout inhibitor is being investigated to determine the effective amount required for successful long term storage. 1,4-DMN has been exempt from having an MRL set by the USA-EPA because it is a naturally occurring chemical in potato and its extreme volatility ensures that a low level of 1,4-DMN would remain on treated potatoes after application. Potatoes treated with DMN retain little or no residue of DMN 30 days after treatment (Jim Zalewski, personal communication). Beveridge (1979) and O'Hagan (1991) pointed out that in commercial stores the residue level of DMN on potatoes can be reduced by a period of airing towards the end of storage due to its high volatility.

In reviewing the literature, relatively little is known about the 1,4-DMN tuber residue level required to control sprout inhibition. However, a recent study was conducted by De Weerd et al. (2010) to identify the lowest residue level that can inhibit sprouting. Using application rates from 0 $\mu\text{L/kg}$ to 56 $\mu\text{L/kg}$ of DMN to treat four varieties of potato tubers, they showed that residue levels were higher and that sprout inhibition was maintained for longer, up to more than 50 days at higher rate applications. Sprouting was no longer inhibited and resumed below 1.4 – 2.7 mg/L for all four varieties. In this study the authors indicated there were many factors that affected tuber residue levels after 1,4-DMN application. These included application method, percent headspace of the treated storage area, storage surface area to potato weight ratio and length of incubation prior to introduction of fresh air and intensity and length of fresh air purging.

Oteef (2008) analysed potato tubers treated with 1,4-DMN and stored for 18 weeks under commercial storage conditions; and the residues in unwashed individual potato samples ranged from 0.63 to 1.16 $\mu\text{g/g}$ fresh weight. Oteef concluded that most of the residues were concentrated in the potato peel. Washing under running water showed no significant difference in residues (0.55 – 1.12 $\mu\text{g/g}$ fresh weight) comparable with the above residues for unwashed tubers, suggesting negligible residues of 1,4-DMN in the attached soil and dust on the surface of the potato tuber. The author suggested that over the storage period 1,4-DMN migrated into the tuber tissues and/or bound onto soil as an unextractable residue. In this study, the effect of oven drying at $75 \pm 5^\circ\text{C}$ on the residues of 1,4-DMN in potato peel (10 g) was investigated and showed that 96% of the residues (4.35 to 0.17 $\mu\text{g/g}$) were removed from the peel after a period of 67 hours.

A study by Knowles et al. (2005) found low residue levels of DMN in aged seed tubers at the end of a 200 day storage period under the highest temperature (9 °C). Furthermore, the tuber residues remaining after 50 days at the three treatment levels (40, 40, 10 mg/kg) were 5.1 ± 0.9 , 2.0 ± 0.2 and 1.5 ± 0.1 mg/kg at store temperatures of 4, 7 and 9 °C respectively, indicating greater loss of 1,4-DMN at higher store temperatures.

It was quoted that the highest residue of 1,4-DMN was 5 µg/kg at about 24 – 28 hours after application when there was no outside venting but after that the residue on the potato began to decline rapidly (2 µg/kg or below) when the store was vented (John Forsythe, personal communication).

CIPC

In 2007, the maximum residue level (MRL) for potatoes treated by CIPC was fixed at 10 mg/kg for human consumption across Europe by Advisory Committee on Pesticides (ACP). In 2009 the European Communities Commission (SANCO/4967) recommended that both CIPC and 3-CA be included in the maximum residue level value (MRL) from 2011 (European-Commission, 2009). However, in recent years, difficulties have been experienced in terms of both the evenness of the application of CIPC and also CIPC residue levels in potato samples, with some exceeding the MRL which resulted in withdrawal from the exporting market and in consumption risk (Noel et al., 2004). There are several possible explanations for this, one is that highest recommended application was exceeded, the second being uneven distribution into the pile of potatoes in the store. It is possible that the dose received by some potato tubers was higher than necessary to control sprouting (Noel et al., 2003; Noel et al., 2002).

CIPC residue levels can vary among potato tubers and are influenced by a number of factors related to the application of CIPC in the potato store and to processing. These factors are summarised below and are supported by the comprehensive literature that has been published on CIPC:

Storage temperature: the conditions in commercial stores have a big effect on the uniformity of distribution of CIPC, good ventilation is particularly important to control the temperature in the store (Kleinkopf et al., 2003). CIPC residues were found to be significantly higher in potato peel stored at 4 °C compared to potatoes stored at 12 °C (Ezekiel and Brajesh, 2007). In addition, Mondy et al. (1992a) observed that the highest

residue levels were found in tubers stored for 6 months at 5 °C compared to those stored at 20 °C. This can be explained by the relatively volatile nature of CIPC at 20 °C.

Application: CIPC residues also vary with the kind of formulation used and method of application (Brajesh and Ezekiel, 2010; Coxon and Filmer, 1985). A study was set up to evaluate the distribution of CIPC among potatoes after applying different formulations: dust powder, emulsifiable concentrate and hot fogging (Noel et al., 2004). The authors concluded that the residue of CIPC on the potato tuber depended more on the formulation applied than to other factors. In this study, it was shown that dust powder formulations seem to be the treatment leading to the highest CIPC residue deposit on the potato tuber compared with emulsifiable concentrate and particularly hot fogging which had a very low residue level of CIPC. Conte et al. (1995) also found that there was approximately 10 times more residue present in tubers treated with powder than tubers treated with aerosol. Wilson et al. (1981) observed high residues of 45 mg/kg following aerosol treatment whereas a study by Mondy et al. (1992 b) showed that potato tubers dipped in a 1% emulsion of CIPC resulted in residues of up to 400 mg/kg in the peel. The application rate has an important role on the residue remaining on the potato tuber, however, it is affected by the storage duration. It was found that the residue concentration was higher immediately after spray application of CIPC at a rate of 30 mg/kg compared to 20 mg/kg but at the end of the storage period this difference was no longer detectable (Mehta et al., 2010).

Storage time: has a substantial effect on the variability of CIPC residue on the potato tuber (Brajesh and Ezekiel, 2010). Tubers stored for 3 months contained lower residue levels of CIPC than those stored for 1 month (Mondy et al., 1992b). Lentza and Balokas (2001) found that the residue levels decreased with increasing storage time. Applying CIPC dust, the mean residues in 16 individual tubers in 10, 28 and 65 days were 3.8, 2.9 and 2.2 mg/kg respectively. The consistent decrease in the residues of CIPC in treated tubers with increasing storage period was also observed by Singh and Kaul (1999) using dust application whereas aerosol application by Conte et al. (1995) did not decrease significantly the residues on peeled potatoes. The residue originally built up on the tuber decreases due to volatilisation, possibly microbial decomposition and also possible chemical changes which progress during storage (Singh and Kaul, 1999; Van Vliet and Sparenberg, 1970).

Tuber location in the store: can lead to differences in CIPC residue levels and this is common for both bulk and box stores even though the patterns of variation differ (Park,

2004). CIPC residue levels in the potato peel were found to be higher in samples obtained from the top and the bottom of the pile, whereas the interior of the pile had intermediate levels (Corsini et al., 1979). However, higher residues of CIPC were observed on potatoes near the bottom of the pile than at the top with a variation of between 3 – 8 mg/kg between the top and bottom (Kleinkopf et al., 1997). Brajesh and Ezekiel (2010) reported that the higher residue level of CIPC was found in tubers stored on the ground floor whereas on the first floor the residue was lower. The authors interpreted that these differences in residues were due to the application of CIPC fog rising through the tubers from the ground floor to the upper layers; therefore a greater amount of CIPC settled on the potatoes in the bottom of the store. In contrast, Baloch (1999) indicated that the highest level of CIPC residue was found to be on the top surfaces with minimal levels on the lower levels in the store. It was concluded that the high residue on the top was a result of fall out of the fog directly on the top surfaces since it is introduced via the ventilation system, which circulates from the bottom to the top of the store. Potatoes stored in piles and treated by aerosol had uneven distribution of CIPC, this discrepancy may be caused by differential air flow in the pile (Conte et al., 1995).

Distribution within the tuber: the distribution of CIPC is different within the potato tuber. Coxon and Filmer (1985) reported that most of the residue of CIPC is associated with the peel and that very little penetration of the chemical occurs beyond the peel layer even after storage period of six months at 10 °C. They reported that losses through volatilisation from the tubers surface were minimal. Study results by Singh et al. (2009) revealed that entry of CIPC particles into the tuber through aerosol treatment (17.5 mg/L of CIPC) is higher at the bud end and that the CIPC can move into the tuber up to a depth of 8 – 12 mm. In addition, the high residue in the peel was found to be in a portion taken from the bud end part of the tuber which could be related to the number of eyes present on the tuber surface. The eyes are known to have higher surface area resulting in higher CIPC deposition (Singh et al., 2009). The residue in the cortex was found to be 10 – 20 times lower than in the peel of treated tubers (Mehta et al., 2010). Mondy et al. (1992 b) showed that the residue in the treated peel was up to 400 mg/kg whereas 10 mg/kg was found in the cortex.

Processing and cooking: CIPC residues were determined in processed potato products (e.g cooked, fried, frozen, crisps, chips and fried oil) (Ezekiel and Brajesh, 2007; Ritchie et al., 1983; Nagami, 1997; Nagayama and Kikugawa, 1992). Processing has been shown to reduce the residue of CIPC in potatoes. Several studies have reported a large difference in

the residue of CIPC in the peel, unpeeled and peeled tuber (Brajesh and Ezekiel, 2010; Mondy et al., 1992b; Coxon and Filmer, 1985). Corsini (1979) reported that the residue of CIPC in peel samples of tuber taken from a large commercial store after aerosol application were fairly high (15 – 85 mg/L), but less than 1 mg/L was found in peeled tubers. It was found that peeling removed approximately 91 – 98% of the CIPC amount in the tuber (Lentza-Rizos and Balokas, 2001). This is expected, because CIPC is surface applied and non-systemic in nature so residue levels are affected directly by the removal of the surface layers during processing (Lewis et al., 1996). Therefore, peeling potatoes is considered sensible before consumption to reduce the hazard of high intakes of CIPC. The active ingredients removed from the potato by peeling is much more than washing (Conte et al., 1995). Some residues of CIPC can be removed during the washing process as evidenced by the presence of CIPC in wash water (Sakaliene et al., 2009; Park, 2004). It was found that up to 45% of CIPC was present in soil adhering to treated unwashed tubers (Coxon and Filmer, 1985). Washing by hand with cold water reduced the residue of CIPC from 3.8 to 2.9 mg/kg of potato treated with dust powder and stored for 28 days, meaning 24% of the CIPC residue was present in water (Lentza-Rizos and Balokas, 2001). However, a study conducted by Wilson (1981) showed that washing under running water reduced CIPC concentration from 45 to 40 mg/L, meaning most of CIPC was not removed by washing under these conditions. Applying a more rigorous washing procedure was found to remove 88% (from 1.6 to 0.2 mg/kg) of CIPC from potato treated with an emulsified solution of 0.1% CIPC (Tsumurahasegawa et al., 1992). Boiling potatoes in water or cooking by steaming resulted in reduced residues in cooked tubers as compared to uncooked tubers due to leaching of CIPC into the cooking water (Mondy et al., 1992a). Lentza-Rizos and Balokas (2001) determined the residue of CIPC in boiling water to be 0.2 mg/kg whereas Ezekiel and Brajesh (2007) reported more than this concentration (1 mg/kg).

3-CA

As previously discussed, a number of studies have produced estimates of the CIPC residue in potato samples, but there is still insufficient data for 3-CA residues in potatoes. That may be interpreted as a lack of a suitable analytical method to extract and analyse 3-CA from potato samples. The most important study to recently address the presence of 3-CA residue in potatoes was a collaborative research review conducted between the University of Glasgow and Sutton Bridge Experimental Unit (Potato Council) (McGowan et al., 2010). Although no information on the analytical method was revealed in this study, the 3-CA residue detected on all potato samples collected from commercial stores was between

0.03 – 0.05 mg/kg for unwashed samples and between 0.02 – 0.22 mg/kg for washed samples.

In reviewing the literature, it was found that immersing CIPC in soybean oil and heating at 180 °C gave rise to a gradual decrease in CIPC with an accompanying increase in the production of 3-CA (Nagayama and Kikugawa, 1992). Hence, it could conceivably be hypothesised that frying potatoes treated with CIPC might be a major factor in causing the presence of 3-CA residue.

Due to the general prevalence of sprout inhibitors, their metabolites and degradation products as residues in potatoes and other environmental samples, it has become essential to routinely determine their residue levels, using appropriate and validated analytical methods.

1.4 General aspects of pesticide residue analysis

In recent decades, considerable progress has been achieved in pesticide residue analysis. A number of reviews have described a wide range of pesticide residue analyses in a complex matrix of food, plant materials and environmental samples with advances in methodology and application (Sherma, 2001; Tekel and Hatrik, 1996; Beyer and Biziuk, 2008; LeDoux, 2011; Chung and Chen, 2011; Llorent-Martinez et al., 2011). Due to the low detection levels required and complexity of the nature of the matrix which holds the intended compounds, attention has been focussed in most of these studies on sample preparation and analytical detection; in particular chromatographic methods for final determination. The differences in the structure and the physiochemical properties of these compounds including parent pesticides and their metabolites, make it difficult to find a single method that can be applied to the analysis of all of these compounds. Generally, determination of the residue levels of any substance in real samples consists of four major and important steps including selecting a representative sample, extraction of the intended substance (mostly using an organic solvent), clean up to remove interfering species and finally the analytical technique to identify and quantify the target substance in the extract. To ensure the acceptability of the analytical method, these various steps should be combined to accomplish maximum recovery of the substance of interest from the sample matrix at the end of the analysis. These important aspects of pesticides residues analysis will be discussed briefly below:

1.4.1 Sampling

The aim of this step is to obtain a representative sample selected from a large population of units for analysis of the compound of interest. The samples should be taken randomly to assure they represent the larger population and that comparisons with maximum residue levels (MRLs) are valid. If residue levels do not exceed the MRLs then there will be no toxicological concern. To detect the exact residue level, it is considered that no degradation has occurred of the target compound. In addition, the selected samples should not be contaminated as this might have an affect on the analysis. In the case of potato samples, the soil adhering is preferred to be present on the selected samples for the analysis purposes. Most often, sealed brown bags are recommended for potato sample collection and packing as this provides the best conditions under which to keep the potatoes fresh until their arrival at the laboratory for analysis. The bags have the additional advantage of preventing contamination. Sampling information should be recorded including the date of collection and position where the potato sample was taken in the store. In addition, the number of chemical applications made, the rate of application and the date of the last sprout suppressant application should be recorded. It is recommended to sample potatoes treated with CIPC 20 days after application, however, recently, samples were taken two days after application (Harry Duncan, personal communication). Generally, environmental samples delivered to the analytical laboratory are stored in a refrigerator (4 °C) prior to analysis and preferably within 24 hours to avoid the effects on the residue level via degradation. However, in practical terms this is often not possible.

1.4.2 Extraction

The extraction process is vital in residue analysis. It begins with sample preparation by homogenising or blending to get good uniformity of the matrix (Melo et al., 2012). This is followed by extraction using solvent to remove the target compound from the other components in the matrix. In most extraction methods, plant samples are homogenised or blended with a solvent like hexane, methanol, acetonitrile, acetone, or dichloromethane in order to transfer the residue from the samples to the liquid solution. In general, selecting a suitable solvent or in some cases a combination of solvents is based on the physiochemical properties of the compound to be extracted and the sample matrix. In the case of multiresidue methods, the solvent used for extraction has to be suitable for the extraction of target compounds that have a wide range of polarities (Tekel and Hatik, 1996). For some extraction methods when analysing fresh vegetable samples using non-polar solvents, salts like sodium sulphate and sodium chloride are added to the sample to remove

the moisture which can have an effect on the accurate determination of the target compound (Paiga et al., 2009). Extraction of the residue from the sample can be performed as a single solvent extraction step or as multiple extraction steps. The efficiency of solvent extraction can be affected by many factors such as time, temperature and agitation. Most of the classic analytical methods used to analyse the residues of potato sprout inhibitors involve using homogenisation, soaking, shaking, sonication, heat refluxing and Soxhlet extraction. These extraction methods are simple and no further instruments or apparatus are required. However, modern extraction techniques have been developed in the field of pesticide residue analysis such as microwave assisted extraction (MAE) (Paiga et al., 2009; Barriada-Pereira et al., 2007) supercritical fluid extraction (SFE) (Kaihara et al., 2002; Tekel and Hatrik, 1996), pressurised liquid extraction (PLE)(Barriada-Pereira et al., 2007; Schuermann et al., 2006) and more recently the QuEChERS (quick, easy, cheap, effective, rugged and safe) method (Chung and Chen, 2011; Lesueur et al., 2008; Schuermann et al., 2006). Although these techniques can overcome the drawbacks associated with using classic methods including the use of large volumes of solvent and being time consuming, from an economic viewpoint their use is more costly than when using straightforward solvent extraction methods.

1.4.3 Clean up

Sample clean up or isolation of the target compound is used in order to reduce the detection limit and to avoid any interference from the sample substrate that can adversely affect the identification and quantification of the target compound (Stajnbaher and Zupancic-Kralj, 2003). Usually, the sample clean up step is accomplished using liquid–liquid partitioning and chromatographic purification. Liquid–liquid partitioning involves partitioning the samples in the presence of aqueous and organic phases so that the lipophilic plant material can be concentrated in the latter. In this technique, salts like NaCl can be added to speed up the separation between the two phases. However, liquid–liquid partitioning is not optimal for some crops which require additional clean up steps (Tekel and Hatrik, 1996). Therefore, in most multiresidue pesticide methods a clean up step involves chromatographic purification using solid phase extraction (SPE) and sorption columns. SPE is a simple technique based on the separation of liquid chromatography and carried out on columns (cartridges) or membrane disks. Most often, silica particles coated with bonded organic material are used as the packing material in the cartridges (Oteef, 2008). Moderately polar to polar compounds can be extracted from non polar solutions onto polar sorbents. Sorption columns include Florisil, alumina, silica gel and carbon

black. These adsorbent columns provide a good clean up only when they are eluted with solvent mixtures of low polarity eluting less polar residues and leaving more polar co-extractives in the column (Tekel and Hatrik, 1996; Ambrus and Thier, 1986). However, in some cases the extensive clean up steps are not recommended in pesticide residue methods due to a number of reasons: the loss of target compounds, they can be time consuming and often they can be costly (Stajnbaher and Zupancic-Kralj, 2003; Menkissoglu-Spiroudi and Fotopoulou, 2004). Therefore, for these reasons and because of the high selectivity and sensitivity of some detectors in liquid chromatography, some extraction methods were developed without adopting clean up steps (Paiga et al., 2009; Ambrus and Thier, 1986).

1.4.4 Analysis techniques

Following extraction and clean up steps, the extract is introduced to suitable analytical techniques for the separation and determination of the analyte in the extract. Numerous analytical techniques have been reported in the literature to analyse pesticide residues particularly the potato sprout inhibitor CIPC in purified extract form. There include colorimetry, (Friestad, 1974; Ferguson and Gard, 1969), thin layer chromatography (Young-Duck and Bergner, 1981; Babic et al., 1998), infrared spectroscopy (Franconi, 1968; Ferguson et al., 1963), gas chromatography (GC) (Hajslova and Davidek, 1985; Beernaert and Hucone, 1991), a combination of gas chromatography-mass spectrometry (GC-MS) (Lewis et al., 1996; Worobey and Sun, 1987; Stajnbaher and Zupancic-Kralj, 2003) and HPLC (Sakaliene et al., 2009; Martindale, 1988).

The colorimetric methods applied for analysis of CIPC are based on acid or alkaline hydrolysis of CIPC to an aromatic amine (3-CA) which is measured spectrophotometrically after coupling with the dyes N-(1-naphthylethylene) diamine dihydrochloride or N-(ethyl-1-naphthyl) amine (Wilson et al., 1981). The drawbacks with the colorimetric methods are that there can be interference from matrix or aniline compounds and that the extra sample preparation is required. It should also be noted that determination of CIPC by colorimetric methods is affected by the amount of 3-CA in the sample. Thin layer chromatography permits fast separation and the limit of the detection is much higher. Both GC and infrared methods require lengthy sample preparation and derivatisation which may be not sensitive at very low residue levels (Corsini et al., 1978). Currently, most of the widely used carbamate and phenylurea pesticides were not directly estimated by GC, mainly due to the stability problems of these pesticides that occur under common conditions with gas chromatography analysis due to their tendency to break down to related phenols and amines on the GC column (Soler et al., 2004; Wilson et al., 1981;

Grou et al., 1983; Paiga et al., 2009). Therefore, the increasing availability of HPLC analysis is nowadays replacing other techniques, particularly for analysing carbamate pesticides and their metabolites in addition to poly aromatic hydrocarbons mainly owing to:

1. Capability and suitability for analysis of a wide range of compounds (low volatility, polar and thermally labile compounds which are stable in the HPLC system) (Hidalgo et al., 1998; LeDoux, 2011; Soriano et al., 2001; Voyksner et al., 1984; Melo et al., 2012; Orejuela and Silva, 2004).
2. Less or no clean up steps required (Fedotov et al., 2004).
3. No derivatisation step hence less time consuming (Lawrence and Leduc, 1977).
4. Decrease in the chance of errors resulting from derivatisation (Lawrence and Leduc, 1977).
5. Producing reproducible responses under various conditions of analysis (Lawrence and Leduc, 1977).
6. High speed, resolution and sensitivity with low detection limit (Sun and Lee, 2003; Lawrence and Leduc, 1977; Lawrence, 1987).
7. High versatility and can be successfully used with various kinds of solvents and columns (Snyder et al., 1988).
8. Appropriate to analyse large volumes (~ 500 μL) rather than the small volumes required for GC analysis (less than 20 μL) (Oteef, 2008).

Due to these advantages of HPLC over other techniques, the focus in this study was toward applying this technique for the residue analysis of the compounds 1,4-DMN, CIPC and its metabolite 3-CA. Therefore, in the end of this part of the literature on residue analysis and before going further to HPLC instrumentation and method development, it would be worthwhile to review some studies of extraction and specifically HPLC analysis of residues of these compounds.

To date there has been few studies reported on the HPLC analysis of 1,4-DMN residues. O'Hagan (1991) started the first work developing a suitable extraction, clean up and quantification method for the analysis of 1,4-DMN in potato residues using HPLC. The method involved homogenising and macerating potato samples with methanol as the extracting solvent followed by filtration and separation in a separating funnel, adding sodium chloride and methylene dichloride. The extract of methylene dichloride was collected and the solvent was evaporated at 24 °C to a volume not less than 4 mL. Finally, the extract was submitted to a clean up treatment using Sep-pak silica cartridges prior to analysis by HPLC.

HPLC analysis of 1,4-DMN residues in potato peel samples was performed recently by Oteef (2008) who conducted a study of several extraction procedures. An HPLC method (TMP/Heat method) was validated that extracted 10 g of chopped peel with 15 mL of mixed extracting solution of ethanol and 2,2,4-trimethylpentane at a ratio of 7:3 (v/v) and in addition contained an internal standard of 2-methylnaphthalene. Heating using a water bath at 50 °C for 15 minutes with occasional swirling was performed and the extract then cooled for 10 minutes prior to centrifuging the aqueous/solvent liquid phase. Two mL of 0.2 M sodium chloride solution was added and centrifuged for 2 minutes. An aliquot of the 2,2,4-trimethylpentane layer was then analysed directly by HPLC. This method proved to have many advantages regarding speed, sensitivity and accuracy for residue analysis of sprout inhibitors in treated potatoes compared to GC analysis. This method was validated according to the procedure used by Knowles et al. (2005) who used hexane instead of TMP; and the 1,4-DMN in hexane layer was quantified by FID-GC.

Several studies have been carried out to determine CIPC residues in potato and environmental samples using HPLC analysis but literature reviews are rarely applied to the determination of CIPC metabolites, in particular 3-chloroaniline. Singh et al. (2009) determined the residue of CIPC in the peel and flesh of processing potatoes using hexane and anhydrous sodium sulphate and Kieselguhr that was mixed and ground with the potato samples using a pestle and mortar. After the clean up step, the extract was filtered and reduced to dryness in glass vials below 30 °C to avoid the loss of CIPC due to volatilisation. The concentrated extract was dissolved in methanol prior to analysis by HPLC equipped with a UV visible detector.

HPLC coupled with a photodiode array detector was used by Sakaliene et al. (2009) to analyse the residue of CIPC in potato samples during storage and processing (washed and

unwashed whole tuber, peel, boiled and puréed tubers). The extraction was performed by homogenising the potato samples (25 g) with 75 mL of a mixture of dichloromethane and acetonitrile (70:30) for 2 – 3 minutes. The mixture was then placed into a centrifuge to obtain the supernatant for analysis.

Direct determination of some carbamate pesticides including CIPC, in water and soil samples was performed using HPLC analysis (Grou et al., 1983). Water extraction was carried out by placing 250 mL sample in a 500 mL separating funnel, adding dilute sulphuric acid to make pH 3, followed by adding 10 g of sodium chloride and finally methylene chloride was added to the solution as the extracting solvent. The extract was dried by passing through a sodium sulphate column then taken to dryness in rotary evaporator prior to HPLC analysis after dissolving the extract in 1 mL of methanol. The soil extraction procedure involved homogenising 50 g with 150 mL of acidic ammonium acetate at 60 °C for 1 hour followed by filtration with a Buchner funnel. The filtrate was placed into a separation funnel and shaken after adding 10 g of sodium chloride. The remainder of the extraction procedure and HPLC analysis was the same as for the water sample extraction.

Recently, HPLC with UV detection have been coupled with modern extraction techniques for the determination of CIPC particularly in soil and water samples. As an example, microwave-assisted extraction (MAE) was used to extract five carbamates (propoxur, thiuram, propham, methiocarb and chlorpropham) from soil and also to study the thermal degradation after heating for 6 minute at 95 °C with different extractants (methanol, dichloromethane, acetone, hexane and water) (Sun and Lee, 2002). Lower recoveries were obtained for all carbamates with less polar extractants and the higher polar extractant showed less degradation of the analyte. HPLC was also coupled with solid phase extraction (SPE) for the determination of pesticides including CIPC in water eluting the concentrated analyte from a disposable SPE cartridge with acetonitrile (Marvin et al., 1990). Recoveries for all pesticides ranged from 84 – 93%.

Most of the standard traditional analytical methods applied at the University of Glasgow to determine the levels of CIPC and 1,4-DMN residues in potato samples are based on GC analysis coupled with flame ionisation detection (Beveridge, 1979; Boyd, 1988; Baloch, 1999). The FID detector is very sensitive to many matrix derived organic compounds in the extract of plant tissue. Therefore and as mentioned above, GC analysis necessitates important steps such as sample clean up and concentration of the extract prior to sample

injection. These steps consume considerable time; therefore, UV detection is accepted as a better alternative to avoid lengthy sample preparation time as only a few species of compounds are responsive to UV light. Taking the above observations into consideration, it is considered to be more practical to use HPLC coupled with UV detection to analyse both the residues of CIPC and 1,4-DMN in the extract of potato samples (Oteef, 2008).

1.5 HPLC method development and validation

1.5.1 Basics and instrumentation

High Performance Liquid Chromatography (HPLC) is one type of chromatography technique besides gas chromatography, thin layer chromatography and supercritical fluid chromatography. HPLC has been extensively used for separation and determination of pesticide residues in foodstuffs and other areas (Torres et al., 1996). A liquid mobile phase is used to separate the analytes in mixtures in solution after interaction with HPLC column under high pressure. In the HPLC column, the mixture is distributed between the stationary phase and the mobile phase. The separation is dependent upon the extent of interaction and affinity between the solute components and the stationary phase. The component that has lowest affinity for the stationary phase will separate first. The mode of HPLC chromatographic action is most often classified according to the nature of the stationary phase and the separation process e.g. reversed phase (RP), normal phase, ion exchange and size exclusion chromatography (Snyder et al., 1988).

Reversed phase (RP) is the most commonly used HPLC separation technique, characterised by hydrophobic interactions with the stationary phase and hydrophilic interactions with the mobile phase depending on the proportion of organic solvent in the mobile phase. Normal-phase chromatography is the opposite of reversed phase chromatography. The stationary phase of HPLC column is strongly polar e.g. silica gel, whereas the mobile phase consists of a non-polar solvent such as hexane and heptane usually mixed with more polar solvent like isopropanol. The stationary phase retains polar compounds on the basis of dipole-dipole interactions. In ionic exchange, the interaction is ionic and the solutes can be separated as ions using an ion exchange resin or bonded silica. Exclusion chromatography is unlike the other kinds of chromatography, the separation is based on the size and shape of molecules in the mixture solution, the bigger the molecules the less they are retained (Lindsay, 1992).

An HPLC instrument is designed to include: a mobile phase reservoir, pump, injector, column, detector and data handling system (see Figure 1:3). The mobile phase in RP-HPLC is commonly composed of water or buffer mixed with various proportions of one or more solvents such as acetonitrile and methanol which have minimum ultraviolet cut off. A high pressure pump is required to force the solvent from the mobile phase reservoir into the column at a constant and reproducible flow rate or pressure. HPLC pumps can be classified into mainly two types: constant pressure and constant flow. The latter is widely used in the majority of current HPLC applications because constant pressure pumps can change the flow rate causing retention data to lack precision and create baseline noise (Lindsay, 1992). Before entering the pump, the mobile phase should be filtered and degassed as any particulate matter and bubbles can affect the pumping action. The elution can be performed either isocratically using a constant composition mobile phase or by gradient elution which involves solvent programming (the mobile phase strength increases during the separation process). In this situation the mobile phase would become less polar and hence the separation time is decreased.

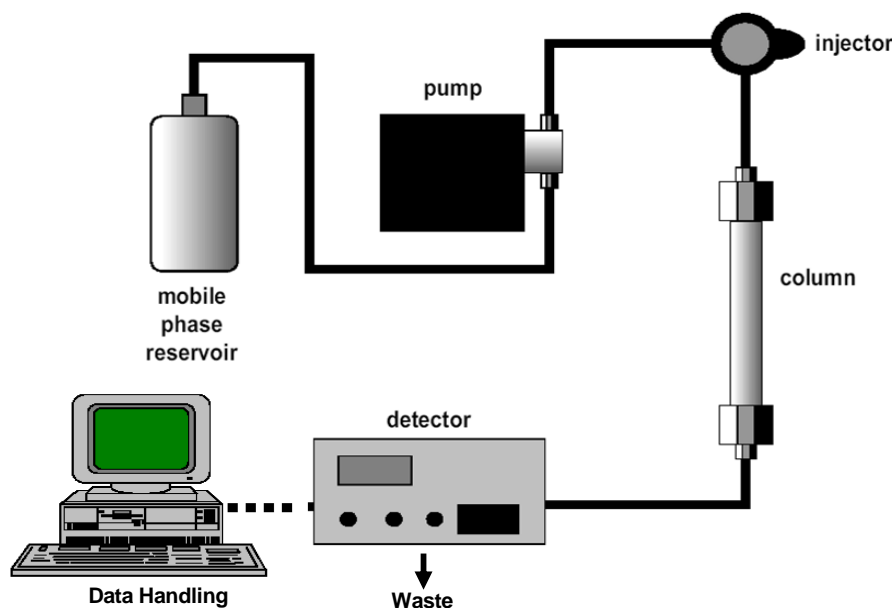


Figure 1:3. Diagram of the general structure of an HPLC system.

Introduction of the sample into the HPLC system is done through the injector either by using injection valves or by automated injection devices. The latter is very common in more sophisticated systems used to analyse large numbers of samples (up to 100). With these devices, the sample is loaded by syringe into the loop sample and mixed firstly in the stream of the mobile phase prior to transfer to the column (Lindsay, 1992). The capacity of injector loop sample ranges from 10 μL to 500 μL , however, the 20 μL is most standard. Filtration is required for the sample before injection, otherwise accumulation of particulate

materials will eventually block the injector, column inlet and column packing, hence more troubles during HPLC system operation can be expected (Snyder et al., 1988).

The column is the heart of the HPLC system and the stainless steel tube is packed with stationary material either polymer or silica that has regularly sized and shaped particles. The stationary phase material is very important as it helps retain molecules on the column whether they are polar, non-polar, ionic, or neutral (Snyder et al., 1988). Silica is a common stationary phase and has active adsorption sites of silanol (Si-O-H) groups bonded with an organic surface layer either C₈ or C₁₈ due to their selectivity and sensitivities for several compounds. The latter is the most popular non-polar phase used as octadecylsilane (ODS). To control the temperature of the column, the majority of HPLC systems contain a column oven, in addition to a guard column which will protect the column from any impurities present in the sample extract.

Detection of the analyte is performed using a suitable detector with a low detection level (e.g. UV, diode array, fluorescence, electrochemical, or mass spectrometry detectors). The most important characteristics required of detectors are; sensitivity, linearity, selective response, low dead volume, cheap and easy usage (Lindsay, 1992). Ultraviolet (UV) detectors are the most widely used coupled with HPLC mainly owing to low cost and the ability of some compounds in the sample matrix to absorb light at one or more wavelengths in the UV range. A conventional UV detector is capable of measuring the absorbance at one wavelength of the solute in the sample, unlike a diode array detector (DAD) which can measure the absorbance at several wavelengths. Using DAD can also provide more identity showing the spectrum of each component in the sample. The variations in the intensity of UV light absorption by each of the components are recorded by generation of an electronic signal for the HPLC chromatogram.

Using the software system as the last component in the structure of HPLC system is important for showing the final data of the peaks of interest in the chromatogram (e.g. retention time and peak area) and optimising method development through instrumental control (increasing the validation for precision and accuracy). Also, the software can make predictions and decisions for improving the separation as a function of experimental conditions (Snyder et al., 1988).

1.5.2 HPLC Method development

Developing an analytical method aims to determine the target analyte in the sample matrix. Before starting method development there are many considerations that should be taken into account such as: the nature of the sample, the aim of the analysis and the availability of HPLC instrumentation. Important information regarding the properties of the analyte of interest should be known, such as chemical structure, UV spectra and solubility. Samples can be in different forms such as solid, solution and mixtures of the analyte with an insoluble sample substrate. Some samples contain interferences affecting the separation efficiency or some components may damage the column; therefore, pre-treatment of these samples is required before injection into the HPLC. The aims of the separation have to be specified whether it is required to identify and determine one chemical, several chemicals (e.g. CIPC and 3-CA) or all of the sample components. Selecting the appropriate detector before starting method development is determined by, for example, whether one component is being measured requiring single detection or whether qualitative analysis is required where universal detection would be preferred (Snyder et al., 1988).

After taking the above into consideration, method development should start with the chromatographic separation step which requires selecting an HPLC method and optimisation of the experimental conditions. Nowadays, different approaches to HPLC method development are used. Reversed phase-HPLC methods are often selected as an initial choice. It is increasingly considered the best separation technique to achieve high resolution, a short run time and better reproducibility of retention time by manipulating the HPLC conditions (Wang et al., 2003; Majors and Przybyciel, 2002). However, other types of chromatography may be appropriate depending on the sample composition.

Separation is the primary objective in routine HPLC methods and aims to resolve all significant components in the sample matrix from each other. The quality of separation is measured by resolution. Resolution (R_s) in liquid chromatography is a measurement used to quantify peak spacing of two adjacent bands in the chromatogram (Dolan, 2002b). Typically, resolution is expressed according to the following equation:

$$R_s = [2 (t_2 - t_1) / (W_1 + W_2)] \dots (1)$$

Where t_1 and t_2 refer to the retention time of two adjacent peaks and W_1 and W_2 peak width measured at baseline (Snyder et al., 1988). This equation (1) is applied to measure R_s when the band peaks are entirely separated. Practically, it is not easy to measure accurately the

baseline bandwidth between overlapping peaks, therefore the bandwidth at half-height (w_1 and w_2) of the peak is the best way of measuring R_s and is expressed in following equation:

$$R_s = [1.18 (t_2 - t_1) / (w_1 + w_2)] \dots (2)$$

If the separated peaks are symmetric, both equations (1 and 2) give the same value of R_s approximately 1.5 which is the case when the valley between two adjacent peaks just touch the baseline. Using this baseline resolution as a minimum value is strongly recommended for quantitative analysis. However, some researchers suggest values of 1.75 to 2.0 during method development of simple mixtures (Snyder et al., 1988; Dolan, 2002b) (see Figure 1:4).

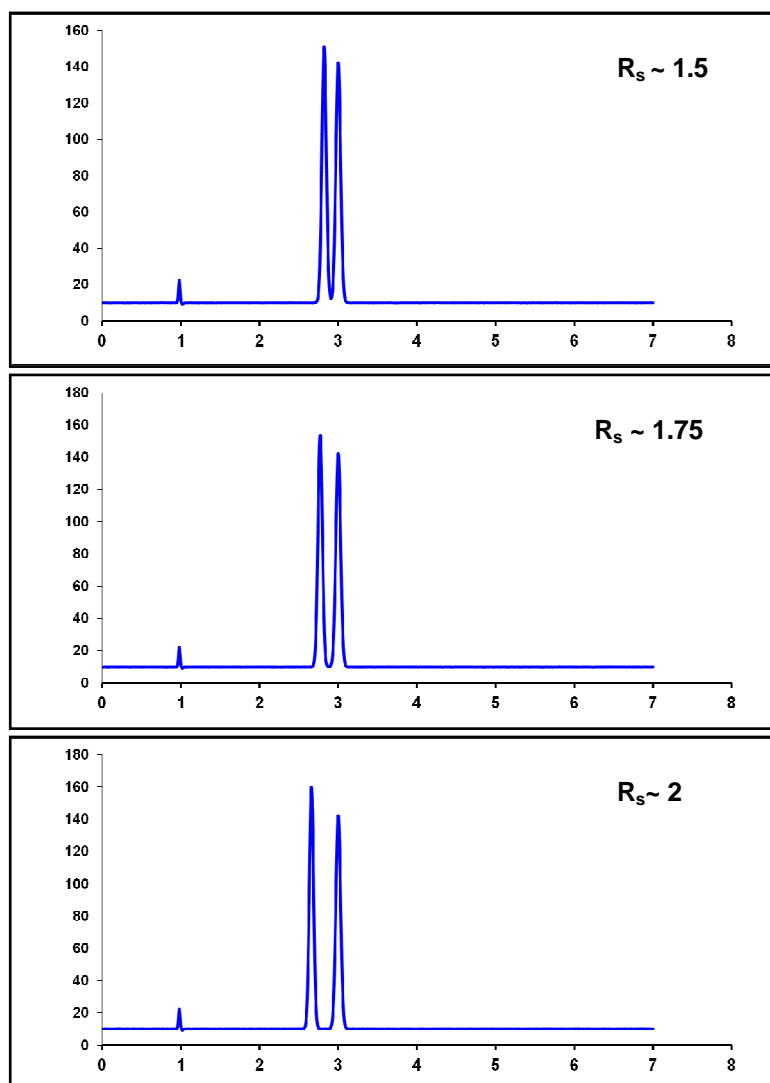


Figure 1:4. HPLC chromatograms showing recommended resolutions between two adjacent peaks.

Resolution can change due to excessive use of the column or fluctuations in the experimental conditions. To control the resolution there are three independent factors

influencing the resolution (R_s); the separation factor (α) of two bands on the column (or selectivity), the capacity factor (k') and the plate number (N) and are expressed in the following basic equation:

$$R_s = (1/4) (\alpha - 1) N^{0.5} [k' / (1 + k')] \dots (3)$$

To obtain a desired degree of resolution, the selectivity factor of separation of the peaks must be greater than one ($\alpha > 1$), the peaks must be retained on the column ($1 < k' < 20$) and the column must develop some minimum number of plates (Lindsay, 1992; Snyder et al., 1988). These factors should be optimised to find the best conditions for a given separation (Fekete et al., 2009). Selectivity optimisation can be done either by changing the temperature or the nature of stationary phase or the nature or composition of the mobile phase. The capacity factor (k') can be controlled by changing the composition of the mobile phase. While, controlling the theoretical plates (N) can be achieved by selecting column length, particle size and or changing flow rate. From a practical view point, all three factors (α , k' and N) are interrelated, by means any change in one from these factors may significantly result in changes in other. Nevertheless, the retention factor should be optimised first then followed by optimisation of the conditions that affect selectivity and the theoretical plates (Lindsay, 1992; Snyder et al., 1988).

Optimisation of HPLC experimental conditions (column packing, temperature and mobile phase) can be approached by trial and error. The initial step is to select an appropriate stationary phase which should provide satisfactory separation factor (Fekete et al., 2009). The choice of a C_{18} bonded phase for reversed phase is preferred. A column with a minimum plate number (approximately 10,000) is also another criterion considered for column performance and separation with suitable peak symmetry. Commercially, there are more than 400 reversed phase columns available, characterised according to the selectivity of five solute-column hydrophobic interactions (Fekete et al., 2009). Generally, there are many factors affecting column separation and efficiency including temperature, particle size, length and the flow rate of the mobile phase (Snyder et al., 1988; Wang et al., 2003).

Column temperature can be an important factor for controlling retention time, selectivity and peak shape in liquid chromatography separation but it can also create problems (Dolan, 2002a). The problem of the thermostating of the HPLC column is mainly for reasons of baseline noise and drifting which should be avoided to get good reproducibility of chromatographic data. The temperature can be varied (0 – 70 °C) to control the selectivity,

usually better resolution will take place at higher temperatures, commonly between 25 and 60 °C (Snyder et al., 1988).

Increasing the length of the column is not preferred, as an increase in retention time gives increased peak broadening. In this case, it is desirable to decrease the plate height equivalent of a theoretical plate by decreasing the particle size of the stationary phase, which leads to an increasing of the separation efficiency of the HPLC column (Lindsay, 1992). This can lead to high back pressures if there is any particulate material in samples.

The optimum flow rate usually depends on the experimental conditions but an optimum flow rate of 1 mL/min is typical for particles of between 3 and 5 μm in size. The column manufacturers and production batches of normally similar column packing from the same manufacturer may vary in retention and separation selectivity as well (Dolan et al., 2002).

Selecting suitable columns and optimising the mobile phase can offer a simple and quick analytical HPLC procedure (Wang et al., 2003). Selecting the mobile phase is very important since it runs the solute with the stationary phase, therefore the solvent in the mobile phase should be pure avoiding any material that can degrade the stationary phase or HPLC apparatus such as strong acids or bases and halides. In RP-HPLC, the use of acetonitrile solvent is the first choice followed by methanol for the initial separation. The advantages of acetonitrile are lower operating pressures, slightly higher solvent strength and applicability for detection in the range of 185 – 205 nm (Lindsay, 1992; Snyder et al., 1988).

The important step after selection of the mobile phase solvent involves selecting the elution mode. Gradient elution should be used with samples which occupy high separation space ($> 40\%$) whereas isocratic elution is possible with samples that occupy less than 25% of the total time or separation space. Separation space is dt/t_G where dt is the retention time of the last peak (t_n) minus the retention time of the first peak (t_1) and t_G is the gradient time or run time (John Dolan, personal communication). Gradient elution is more expensive than isocratic elution which is preferred for simple samples containing less than 10 components (Schellinger and Carr, 2006).

Mobile phase composition plays an important role in improving the resolution and the runtime in particular, changing the percentage of the organic solvent changes the peak spacing. Changing the pH of the mobile phase may also have a major effect on peak

spacing particularly if the sample contains acidic or basic compounds. The final HPLC method should be carried out with a run time that is as short and practical as possible (10 – 20 minutes) allowing a wide range of samples to be analysed (Dolan et al., 1998; Snyder et al., 1988).

1.5.3 Validation of an analytical method

The applicability of analytical methods is assessed by a validation process. Validation is the formal and systematic way to demonstrate the suitability of a developed method for testing the analyte to provide useful analytical data within defined limits (Maldener, 1989). Method validation studies comprise the overall procedure established during method development including sample preparation, analysis and the assessment of the results. The applicability and the requirements mainly depend on the analyte being tested, the analytical method used and the area of application of the method. Quality control procedures for pesticide residue analysis were provided by European Commission guidelines in 2006 (SANCO/10232) for acceptance of a method (European-Commission, 2006). The most common validation parameters for the analytical methods are discussed below.

1.5.3.1 Selectivity and specificity

Selectivity is the ability to separate the target analyte from interferences present in the sample. It is considered the most important parameter in the analytical method validation to provide accurate analyte measurements. Commonly, the term selectivity is used interchangeably with the term specificity. Actually, when the method is poorly selective, a serious mistake is made by describing it as specific. Therefore, one should distinguish between these two terms. Selectivity refers to the ability of the method that can produce responses for a number of analytes in the complex matrix and discriminate the response of a single analyte from the other (Vessman, 1996). Whilst, specificity describes the method that produces a response for only one single analyte. The International Union of Pure and Applied Chemistry (IUPAC) recommended the promotion of the selectivity concept and settled the problem by expressing the idea that “specificity is the ultimate of selectivity” (Denboef and Hulanicki, 1983; Vessman et al., 2001). Analytical chemists in chromatography, therefore, should use these two terms carefully and selectivity should be given top priority in all analytical method developments (Aboul-Enein, 2000). Usually, with using UV detection in HPLC analysis the term of selectivity is very common since it can detect many components present in a sample. The selectivity should be tested against all components present in the sample matrix by using a blank sample with and without the

analytes. These components or interferences have to be separated with acceptable resolution ($R_s > 1.5$) (Maldener, 1989).

1.5.3.2 Accuracy and precision

The accuracy of an analytical method refers to the closeness of the measured value obtained to the true value. Practically, no measurement process is ideal, therefore, the true or actual value can not be exactly known in any particular measurement. Certified reference materials (CRMs) can be used to assess the accuracy of the measurements determining the difference between the measured value and the true value and then to estimate the size of the actual error. CRMs are “materials or substances, one or more of whose property values are certified by a procedure that establishes traceability to an accurate realisation of the unit in which the property values are expressed and for which each certified value is accompanied by an uncertainty at a stated level of confidence” (King and Grp, 2003). Quantitatively, the accuracy and the precision are essential to assess the associated errors in the analytical method (Manoli and Samara, 1999). Thus, it is crucial to identify the sources of errors affecting the accuracy and subsequently to find a better procedure to remove and reduce the impact of these errors.

Experimental errors are classified into three major types; gross, systematic and random errors. Gross errors are defined as the errors causing damage to the experiment and require a new experiment. Systematic errors are that the same errors remain constant for the measurement repeated under the same conditions. The term bias is used to describe a systematic error. Normally, the bias of a measuring instrument can be calculated by the observed value that is described as being biased positive or negative when a systematic error is present. Some sources of systematic errors include spectral interferences and standard preparation. Random errors can be defined as the errors which vary randomly when replicate measurements are carried out under identical conditions. This type of error is inescapable and requires the utmost of care to minimise. Random errors affect the precision, whereas both random and systematic errors influence the accuracy. Another parameter incorporating random and systematic errors is called uncertainty. It is commonly used to describe a realistic range within which the true value of the quantity being measured is expected to lie (Miller and Miller, 2005; Currell, 2000). Sample preparation is the main parameter affecting uncertainty measurement (Meyer and Majors, 2002).

The precision of an analytical method is defined as the degree of an agreement among individual tests obtained when the method is applied to multiple sampling of a

homogenous sample. It is usually expressed in terms of standard deviation (SD) or relative standard deviation (RSD%) for more than five replicate measurements of the standard at low, mid and high concentrations. Four types of precision can be characterised. Repeatability (instrument precision) is evaluated by repeated measurement of the same sample to test the efficiency of the instruments. All instrumental measurements produce some random error or noise which is difficult to remove. However it can be evaluated by suitability testing. The second type is repeatability (intra-assay precision) which is assessed by repeating sample analysis in one laboratory by one analyst using the same conditions. The third type is intermediate precision obtained using the same laboratory and analytical procedure under different operating conditions. Lastly, the most important type of precision is reproducibility when analysing the same solution under different conditions including different laboratories, analysts and instruments (Green, 1996).

1.5.3.3 Linearity and range

The linearity of an analytical method refers to the ability to obtain results either directly, or after mathematical transformation proportional to the concentration of the analyte in the sample within a given range (Shabir, 2003; Chandran and Singh, 2007). Linearity is established by measuring the instrument response of a sufficient number (at least five) of standard solutions in the expected range of the analyte. It is estimated by the equation of the regression line ($y = ax + b$) by plotting concentrations (x) versus the response (y) (Caldas et al., 2009). Some distributed errors are expected to be associated with the regression line. The error source from the measured response is more than the error in the preparation of a sample concentration. Typically, the correlation coefficient is used to express the acceptability of the linearity of the regression line (Chandran and Singh, 2007; ICH, 1994). However, according to different views in the literature, there is a problem in the terminology used for linearity criteria. Authors refer to five different expressions for the linearity criteria including r , r^2 , correlation coefficient, correlation coefficient with r and correlation coefficient with r^2 . Statistically, the Pearson Product-Moment Correlation Coefficient (PMCC) is typically denoted as r . In the case of a straight line graph, the value r^2 is the same as the coefficient of determination and denoted as R^2 which is calculated from the regression line of the calibration curve provided R^2 as a decimal by Excel, but is given as percentages if multiplied by 100. The value of r^2 is always slightly smaller than r (Miller and Miller, 2005). The coefficient of determination R^2 (r^2) explains the variation from the regression line as a percentage. The total variability is expressed by the variability that can be explained from the regression line and the remaining variability is due to other

unexplained factors. For example if r^2 is 89% that means 89% of the variability of the response of y from the regression line can be explained and 11% of the remaining variability is unexplained.

Linearity criteria are reported as a mixture of correlation coefficient of r and coefficient of determination R^2 (r^2) values. Typically, a correlation coefficient of more than 0.995 is considered acceptable for the analysis of biological samples for HPLC assay (Arnoux and Morrison, 1992). While for an HPLC method of pharmaceutical samples at low levels the correlation coefficient should be ≥ 0.98 (Green, 1996). The linearity specification for autosampler performance is acceptable when r^2 is 0.998 or more (Hall and Dolan, 2002; Shabir et al., 2007). Whereas under most conditions, the correlation coefficients according to Chandran and Singh (2007) should be greater than 0.9999. In analytical practice, calibration curves with correlation coefficient r values greater than 0.99 are relatively common (Miller and Miller, 2005).

The range of the method is the interval between the upper and lower levels of an analyte in the sample with acceptable accuracy, linearity and precision (Shabir, 2003; Chandran and Singh, 2007). The range is estimated on either a linear or nonlinear response curve, using the data of the linearity studies and the intended application of the method (Green, 1996). Misinterpretation of the determination of the range can be avoided by plotting the concentration or (log concentration) either against the deviation from the regression line or against the ratio of response to concentration. A non linear calibration may be required in a specific analytical method but mostly a linear type is chosen (Chandran and Singh, 2007).

In chromatographic measurements, three calibration curve methods are used to quantify the analyte accurately: standard addition, external standard and internal standard methods (Wieling et al., 1992). The standard addition method is practically suitable to samples with an analyte concentration close to the sensitivity limit to solve the matrix effect problem. The drawback of this method is that each sample must be analysed many times and it is suitable for measuring only a small number of samples. In the external standard method, a compound present in pure solution is analysed separately from an unknown sample under the same conditions. However, this method has some disadvantages, because each step must be controlled regularly (Wieling et al., 1992). The use of the internal standard method is very common to achieve precise results in environmental applications. It can decrease the contribution of systematic errors to the total errors of the determination (Ostroukhova and Zenkevich, 2006). The internal standard can be added before sample pre-treatment to

improve the reproducibility of the analytical method thus eliminating the variance of the injection volume in the chromatographic system. In addition, it can correct for any losses of the analyte during sample preparation. In some cases, adding the internal standard can be done immediately before the chromatographic analysis rather than before sample preparation but this can only correct for analyte loss associated with the chromatographic measurements not the entire of the analysis procedure (Ostroukhova and Zenkevich, 2006). The internal standard should be selected to mimic the analyte, thus important criteria are considered when choosing the internal standard such as its peak completely resolved, same chemical properties as the target analyte, its retention time close to that of the analyte, detectable under the same conditions as the analyte and absent from the original samples. However, using an internal standard may be associated with some interference that may cause some measurement errors. Therefore, to achieve the best results, the analytical method should be examined with and without an internal standard in order to ensure its suitability for use (Wieling et al., 1992; Aboul-Enein, 1998).

1.5.3.4 Limit of detection and limit of quantification

Limit of detection (LOD) is the minimum concentration of the analyte that can be reliably detected and distinguished from zero (or the noise level of the system), but not quantifiable, whereas limit of quantification (LOQ) is the lowest level of an analyte in a real sample that can be quantified with acceptable accuracy and precision under stated experimental conditions (Caldas et al., 2009; Chandran and Singh, 2007). There are different approaches to determine LOD (commonly but incorrectly called sensitivity) and LOQ values (Armbruster et al., 1994). Most often, LOD and LOQ can be determined based on the signal to noise ratio of the analyte that produces a response 3 and 10 times respectively greater than the noise level of the detection system. Another approach uses the relation $3.3 \text{ SD}/S$ to calculate LOD and $10 \text{ SD}/S$ for LOQ where SD refers to the standard deviation of the detector response and S is the slope of the calibration curve. In the case of LOD, the value 3 usually is applied by many analysts instead of the value 3.3, equivalent to the probability of the error as 7% rather than 5% (Chandran and Singh, 2007; Oteef, 2008). The standard deviation can be calculated as the standard deviation of replicate blank sample responses converting the peak area response to concentration. This method is not desirable in most chromatographic measurements because the peak area response is not measurable in most blank samples. In HPLC method validation, the approach selected to calculate the LOD and the LOQ has to be fixed to avoid discrepancy (Vial and Jardy, 1999). Statistically, the easiest method to measure the limit of detection is through the

regression line of a low range of standard concentrations (Miller and Miller, 2005; Oteef, 2008). The LOD gives a signal (peak area) equal to the blank signal (Y_B) plus three standard deviation of the blank signal ($3S_B$) as in the following equation (see Figure 1:5):

$$\text{Limit of detection of the peak area} = Y_B + 3S_B \dots (4)$$

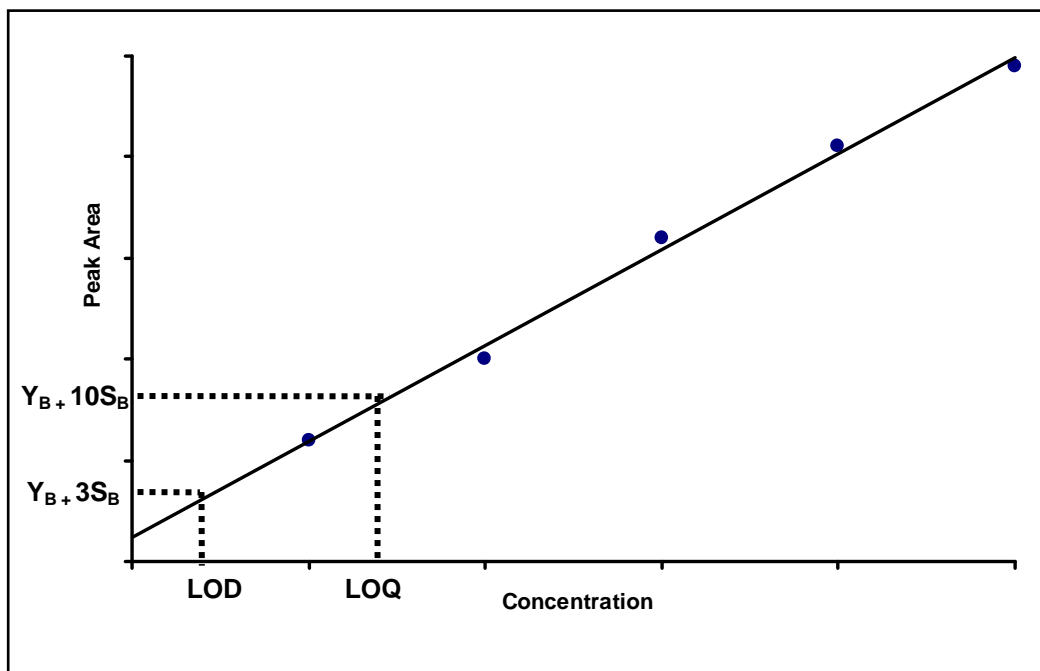


Figure 1:5. Calculating the LOD and the LOQ from the calibration curve depends on the standard deviation of the peak area.

The calibration curve is plotted at low range concentrations in the Microsoft Excel[®] sheet, the intercept and standard deviation of the regression line are replaced by Y_B and S_B respectively to calculate the LOD peak area. In the same manner, the LOQ peak area can be determined by replacing $3S_B$ with $10S_B$. It is better to express the values of the LOD and the LOQ by concentration unit.

In chromatographic analyses, an alternative method to calculate LOD and LOQ is through determining the standard deviation of the response of replicate injections of standards at low concentrations. The LOQ is confirmed by analysing a number of samples known to be near or prepared at the quantification limit. Usually, five to ten times of the minimum detectable quantity value can be injected for quantitative measurement (Lawrence and Leduc, 1977). In order to make an accurate evaluation of the residue in the real sample, the limit of detection of the compounds in the sample matrix should be the same as that obtained for a pure standard solution.

1.5.3.5 Recovery

Quantitative analysis is used to determine accurately the amount of analyte. A recovery study is an important factor in the validation to evaluate the accuracy of the analytical method (Caldas et al., 2009). It is a measure of the efficiency of the analytical method to separate and determine the analyte from the sample matrix after extraction and analysis achieving high recovery with minimum matrix interference at the final measurement step. Recovery has been defined by the IUPAC as “the proportion of the analyte quantity, present or added to the analytical portion of materials tested, which is extracted and presented for measurement” (Thompson et al., 1999). Commonly, in pesticide residue analysis, recovery studies are performed on a blank sample spiked with the analyte of interest, tested at different fortification levels. Generally, there are more than five replicates for each sample at different spiking levels which should be in relation to either the limit of detection of the analytical method or the maximum residue levels (MRLs) of the pesticide in a specified food sample. It is important to obtain high recoveries (close to 100 %) with good precision and small changes in the experimental conditions should not affect the robustness of the recovery values (Wieling et al., 1993). Most often, recoveries of organic compounds are acceptable in the range of 70 – 110% (Linsinger, 2008). Recovery values vary depending on many factors including the sample matrix, sample preparation procedure, properties of the analyte of interest and its concentration. Poor recovery rates can be presumably caused by extraction procedure, evaporation, adsorption and degradation (LeDoux, 2011). Some recoveries exceed 100% but this may be due to some variability during the extraction (e.g. presence of water in the potato peel) or HPLC analysis interferences (Linsinger, 2008; Wieling et al., 1993).

After optimisation and validation of the analytical method produces high recovery rate, it can be applied to real samples for quantitative determination of the analyte of interest. Some laboratories use the recovery values to correct values and others do not (Thompson et al., 1999).

1.6 Aims and objectives

In the UK, due to the increasing concern over the toxicity of chlorpropham (CIPC) and its application in potato stores, there is a requirement to find alternative potato sprout inhibitors to be used. Attention is being given to the naturally occurring chemical 1,4-DMN which is currently used in many countries around the world. Prior to the introduction of this sprout inhibitor to the UK for commercial use, it is important to investigate its fate in potato samples for human consumption and subsequently in the environment. The main aim of this study is to investigate the level and the behaviour of 1,4-DMN, CIPC and its degradation product 3-CA in potato samples, potato washing water and soil. Therefore, the work in the chapters of this thesis includes:

1. Development and validation of isocratic HPLC–UV methods including:
 - a. Developing methods for the analysis of 1,4-DMN and its internal standard 2-methylnaphthalene (2-MeN) using acetonitrile for both the mobile phase and standard preparation.
 - b. Testing three HPLC systems with the aim of selecting the best system for the remainder of this study.
 - c. Developing methods for the analysis of 1,4-DMN and 2-MeN and the simultaneous determination of both CIPC and its degradation product of 3-CA using methanol to overcome the problem of the acetonitrile shortage at the time of the performance of this part of the study.
2. Prior to developing analytical methods for the quantitative measurement of the residues of the studied compounds in potato washing water and soil samples, important preliminary work required the investigation of:
 - a. Solubility, degradation and quantitative analysis of 1,4-DMN, 2-MeN, CIPC and 3-CA in water solutions.
 - b. Testing their potential adsorption on laboratory ware that is commonly used for collecting samples and for analytical determination.

3. Developing and validating a new analytical method for the determination of CIPC in potato samples using methanol as the extracting solvent to overcome the acetonitrile shortage.
4. Evaluation of a new CIPC method to include 3-CA, investigating different parameters including potato variety, extracting solvent, extraction method, spiking procedure and different treatments for potato samples, with the aim being to understand how 3-CA is held on the potato peel thus improving the extractability of 3-CA.
5. Determination of both CIPC and 3-CA in commercial potato samples, investigating the effects of fogging temperature and number of CIPC applications in potato stores on CIPC and 3-CA residues in treated potato samples.

Chapter 2: Routine methods and preliminary assessments

2.1 Routine methods

2.1.1 Preparation of stock standard solutions

Analytical reagents were used from 1,4-dimethylnaphthalene (95%), 2-methylnaphthalene (97%), chlorpropham (95%), 3-chloroaniline (99%) and propham (99%) which were purchased from Sigma-Aldrich Chemi GmbH (Germany). Stock solutions of 10 000 µg/mL of each were prepared by dissolving 1 g in 100 mL each of acetonitrile, methanol and hexane (HPLC grade, Fisher UK). These individual stock standard solutions were stored in a refrigerator and used to prepare the working solutions at different concentrations.

2.1.2 HPLC systems

The main HPLC system used comprised a GILSON[®] 234-auto sampler, Cecil 1100 Series pump, Phenomenex[®] Security Guard[™] (part no. KJO-4282) guard column with analytical column Phenomenex[®] (ODS-2 250 mm x 4.6 mm 5 µm Sphercclone) and a Thermo Separation SpectraSERIES UV100 detector coupled with Dionex Peaknet software. A column oven (LaChrom, Merck L- 7350) was connected with a cooling system (Techne, Tecam[®] R 4-2) to control the column at 25 °C temperature. Other HPLC systems were also used as described in Section 3.2.2.2.

2.1.3 Preparation of the mobile phase

The mobile phase for HPLC analysis was prepared from organic solvent of acetonitrile or methanol and water (v:v%). The water used for preparation of the mobile phase was supplied from a Elga Purelab Option deioniser model LA613, then filtered through a Supor[®]-200 membrane filter (47 mm 0.2 µm). The mobile phase was degassed either using an ultrasonic bath (Camlab CamSonix C425) or helium gas.

2.1.4 Method validation

The HPLC analytical method was validated through the assessment of different parameters:

2.1.4.1 Precision

The precision was assessed by repeated injections of at least ten replicate injections of a standard solution. The precision was calculated through the peak area by determining the relative standard deviation (RSD%) as follows:

$$\text{RSD\%} = (\text{SD}/\text{M}) * 100$$

Where SD is the standard deviation of the peak area and M is the mean of the peak area.

2.1.4.2 Linearity

A set of standards at different concentrations was prepared and injected. Linearity was evaluated according to the relationship between the peak area of the compound and its concentration. Excel[®] software was employed to plot the calibration curve for each compound in the solution. From the regression line, the coefficient of determination (R^2) was obtained to statistically assess the linear relationship.

2.1.4.3 Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of compounds in solution were calculated by two approaches, the first approach was by ten replicate injections of a single solution as following:

$$\text{Peak area for LOD} = 3 * \text{SD}$$

$$\text{LOD} = \text{Peak area for LOD} * (\text{Conc.} / \text{M})$$

$$\text{Peak area for LOQ} = 10 * \text{SD}$$

$$\text{LOQ} = \text{Peak area for LOQ} * (\text{Conc.} / \text{M})$$

Where SD and M are the standard deviation and mean of the peak area respectively and Conc. is the concentration of the solution injected.

The second approach to determine the LOD and the LOQ was based upon the statistical data from plotting the calibration curve in the Microsoft Excel[®] sheet at the lowest range of the concentrations (0.02 – 0.1 µg/mL). These statistical data consist of the intercept, slope and the standard deviation of the regression line (SD), after calculating the LOD and the

LOQ peak area depending on the above information of the regression line, the LOD and the LOQ were determined according to the following equations:

$$\text{LOD Peak area} = \text{intercept} + 3 \text{ SD}$$

$$\text{LOD} = (\text{LOD Peak area} - \text{intercept}) / \text{slope}$$

$$\text{LOQ Peak area} = \text{intercept} + 10 \text{ SD}$$

$$\text{LOQ} = (\text{LOQ Peak area} - \text{intercept}) / \text{slope}$$

The statistical data of the regression line (see Figure 3:12 and Table 3:9) were summarised as an example in the table below:

Table 2:1. The statistical data of the regression line obtained from the Excel® sheet to determine the LOD and LOQ values of 1,4-DMN from the calibration curve in the range 0.02 – 0.1 µg/mL solution of 1,4-DMN.

Statistical data	1,4-DMN
Slope	32892210
Intercept	51446
Line SD	60918
Peak area of LOD	234199
Peak area of LOQ	660622
LOD (µg/mL)	0.0056
LOQ (µg/mL)	0.0185

2.1.5 Preparation of potato samples for analysis

Potato tubers were randomly selected from bags which were obtained from UK stores that had received CIPC application. Washing for two minutes under cool running tap water was carried out to remove the soil and any CIPC that may be adsorbed on to the soil. After air-drying, the weight of each potato tuber was recorded using a top pan balance, each tuber was peeled with a stainless steel peeler and the weight of the total peel was recorded. Using a kitchen knife and chopping board, the peel of the tuber was chopped into fine pieces and carefully mixed to obtain good homogeneity. Ultimately, a subsample was taken for extraction.

2.1.6 Soxhlet extraction

The peel was placed into a cellulose thimble, which contained 10 g of the drying agent sodium sulphate to remove the water from the potato peel. The thimble was plugged with cotton wool and placed into a Soxhlet extraction unit prior to extraction with 150 – 200 mL of solvent. The peel was extracted for approximately two hours after the first reflux. The heater was then switched off but the cooling water was left running for 20 minutes to allow the extract to cool. For HPLC analysis, the extract in the round bottom flask was quantitatively transferred to a volumetric flask and made up to volume, then filtered through a 0.2 µm PTFE (Teflon) membrane syringe filter prior to analysis. For GC analysis, the extract was concentrated using a rotary evaporator (Büchi Rotavapor RE111) coupled to a water bath (Grant JB2 thermostat) at 35 °C to evaporate the solvent. The concentrated extract was transferred quantitatively to a volumetric flask (2 mL) and made up to volume.

2.1.7 GC analysis

Analysis was performed on a Hewlett Packard HP 5890A gas chromatography with a Flame Ionisation Detector (FID), HP 7633A auto sampler unit and DB-1 column (30 m, 0.53 mm i.d., 1.5 µm film thickness). The oven program was started at 40 °C for 4 minutes then increased at 55 °C/min up to 175 °C and held for 10 minutes, then 15 °C/min up to 230 °C and held for 10 minutes. The injector temperature was set at 220 °C and the detector at 250 °C. An internal standard of 100 µg/mL of IPC was used to overcome the variability of injection volume caused by the autosampler. A mixed standard of 100 µg/mL of CIPC, IPC and 3-CA prepared in hexane was injected in duplicate, setting the injection volume to 2 µL. The retention times were approximately 6, 10 and 15 minute for 3-CA, IPC and CIPC respectively at a run time 18 minute.

2.1.8 pH measurements

A Mettler Delta 320 pH meter coupled with plastic bodied pH electrode (Fisher brand) was employed to measure the pH of all standard and extract solutions. The pH meter was calibrated by buffer solutions of pH 4 and 7, which were prepared by dissolving one tablet in deionised water and made up to 100 mL to produce a buffer solution of each pH at 20 °C.

2.1.9 Estimation of the water weight percentage in potato peel

The water percentage was measured by weighing fresh peel into a crucible which was placed in an oven (Gallenkamp, Hotbox Oven Size 1) at 100 °C overnight. Later, the dried peel was weighed to calculate the amount of water lost.

2.1.10 Preparation Tenax traps

Tenax traps were prepared using glass tubes (6 mm o.d., 3 mm i.d and 105 mm length) which were rinsed with acetone then toluene prior to immersion in a 5% solution of hexamethyldisilane (HMDS) in toluene for 15 minutes. HMDS was used to prevent any adsorption of compound onto the glass by deactivating any bonding sites. Next, the tubes were rinsed with toluene followed by acetone then dried in an oven at 100 °C for 15 minutes. After cooling, each tube was packed with a 2 cm bed length of Tenax GC resin and conditioned under a flow rate of nitrogen at high temperature (300 °C) for 2 hours in an oven to remove sorbed volatiles or any impurities. The tubes were allowed to cool under nitrogen and then removed from the oven and the ends of the tubes sealed with PTFE tape and aluminium foil until use (Park, 2004). After use, these Tenax traps were washed with 150 mL of ACN refluxing in a Soxhlet apparatus for 4 hours, then dried in oven at 110 °C overnight. After cooling, they were sealed with Teflon tape and aluminium foil and stored in the fridge at 4 °C until reuse.

2.2 Preliminary assessments of the study

2.2.1 The accuracy and precision of pipetting

In order to validate the accuracy and precision of the pipettes required to prepare standard solutions in this study, 10 aliquots of 1 mL water and the same for acetonitrile at ambient temperature were put into a Quick fit container and weighed on an analytical balance using a glass pipette type B mL (± 0.015) and micro pipette (P1000 Gilson). The accuracy was measured through the bias% by converting the mean weight of the aliquots to true volume at the test temperature (17 °C). The densities of water and acetonitrile at this temperature are 1.0022 and 0.786 g/mL respectively. The precision of the pipette was measured as the relative standard deviation (RSD%). The calculations are shown below:

$$\text{Bias\%} = \frac{(V_t - V_o)}{V_o} * 100$$

$$V_t = M_w/D$$

$$\text{RSD\%} = (\text{SD}/\text{Mw}) * 100$$

Note:

V_t : true volume

V_o: indicated volume (1 mL)

M_w: mean of replicate weights

D: the conversion factor for density at given temperature

SD: standard deviation of replicate weights (n = 10)

The results are shown in Table 2:2.

Table 2:2. Bias% and RSD% values for the pipettes.

Pipette	Bias%		RSD%	
	Water	ACN	Water	ACN
Glass Pipette 1 mL B (± 0.015)	0.57	0.55	0.45	0.08
Micro Pipette (P1000 Gilson)	– 0.14	– 2.49	0.23	1.13

Bias% and RSD% values in this table were compared with the bias% and the RSD% specifications of the micro pipette (P1000 Gilson) for the calibration of volumetric ware which should be ± 0.8 and ≤ 0.15 respectively using distilled water whilst the tolerance (limit of bias%) of the glass pipette was $\pm 1.5\%$ (BSI, 1986). Experimentally, the glass pipette was shown to have higher accuracy and precision than the micropipette for acetonitrile, but using the micropipette gave more accuracy and precision for water. This could be due to the differences in the physical properties e.g. the viscosity and the density of water compared to acetonitrile.

2.2.2 The accuracy and precision of standard preparation

The accuracy and the precision of preparation of standard solutions were examined by preparing five solutions of the same concentration (1 µg/mL) of a mixture of 1,4-DMN and 2-MeN using a glass pipette type 1 mL B (± 0.015) and using a micropipette (P1000 Gilson). These solutions were injected into three HPLC systems and then the accuracy and

the precision were measured for each compound calculated as the relative bias% (the difference between the two types of pipettes) and RSD % as shown below:

$$\text{Relative bias\%} = \frac{[\text{MPA using micropipette} - \text{MPA using glass pipette}]}{[\text{MPA using glass pipette}]} * 100$$

$$\text{RSD\%} = \frac{\text{SD}}{\text{MPA}} * 100$$

Where MPA is mean peak area for five replicates.

Table 2:3. RSD% and Bias% values of 1,4-DMN and 2-MeN on three HPLC systems in solutions prepared by glass pipettes and micropipettes.

HPLC SYSTEM	RSD% (n = 5)				Bias%	
	Micro pipette		Glass pipette		2-MeN	1,4-DMN
	2-MeN	1,4-DMN	2-MeN	1,4-DMN		
Hitachi DAD (auto sampler)	0.44	0.31	0.68	0.69	- 2.35	- 2.05
SpectraSERIES UV100 (manual injector)	2.89	3.18	0.67	1.01	- 2.67	- 2.64
SpectraSERIES UV100 (auto sampler)	0.31	0.38	1.30	0.64	- 2.56	- 1.80

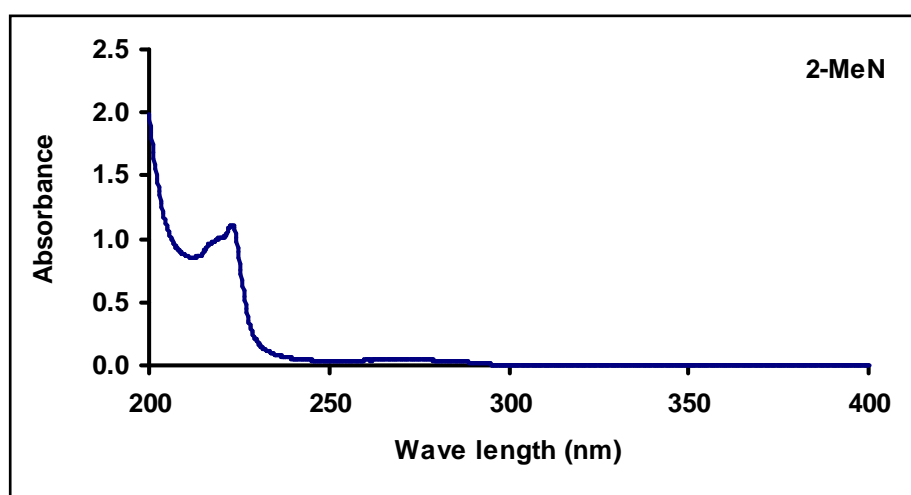
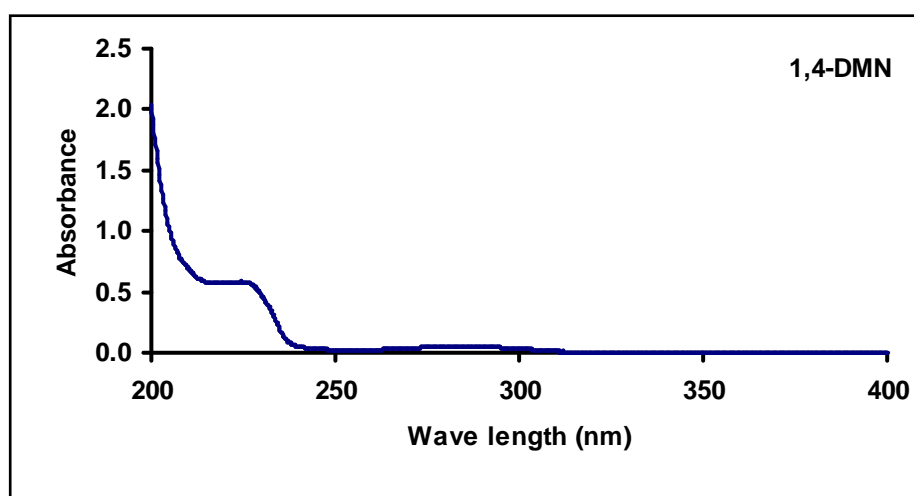
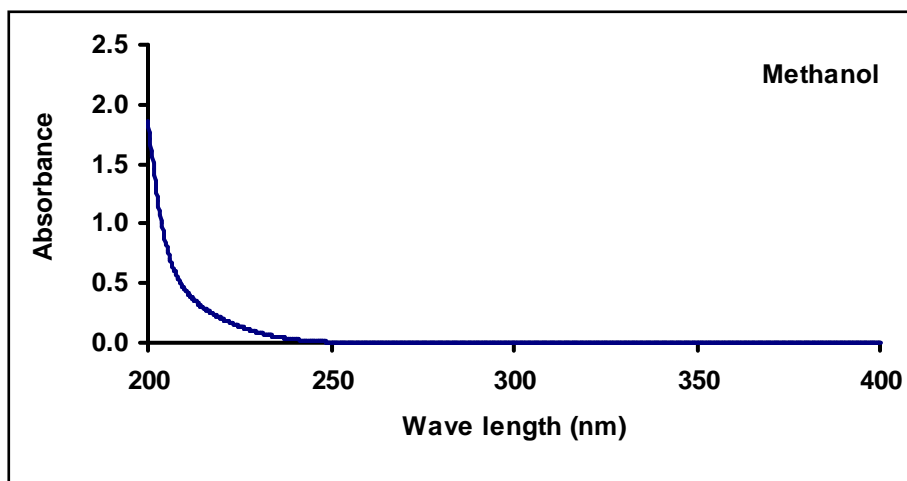
The RSD% values indicated a high precision particularly with the HPLC systems consisting of an auto sampler injector compared to the manual injector. The Bias% values showed that there is little variance between the bias% of the peak area of each compound on each system. These values largely refer to systematic errors during the preparation using volumetric glassware because the micropipette delivers a small volume.

From these above experiments the glass pipette was chosen for preparation of the standards in this study.

2.2.3 Determination of maximum absorption of studied compounds

A UV-VIS Scanning spectro photometer Shimadzu UV-2101PC was used to measure the wavelength (λ_{max}) at maximum absorbance for 1,4-DMN, 2-MeN, CIPC, IPC and 3-CA in both solvents of methanol and acetonitrile in the range 200 – 400 nm. The λ_{max} is required to detect these compounds for HPLC analysis and to ensure there is no absorbance of solvents in this range. A 1 $\mu\text{g/mL}$ standard of each of 1,4-DMN and 2-MeN and 5 $\mu\text{g/mL}$ of each of CIPC, IPC and 3-CA was prepared and analysed.

From the spectra as shown in Figure 2:1, it can be seen clearly that there is a broad band with a shoulder of these compounds in the methanol solution (cut off 205 nm) which caused overlapping of the peaks for all of the compounds; therefore it was difficult to identify the maximum UV absorbance and optimum wavelength λ_{max} of these compounds in methanol.



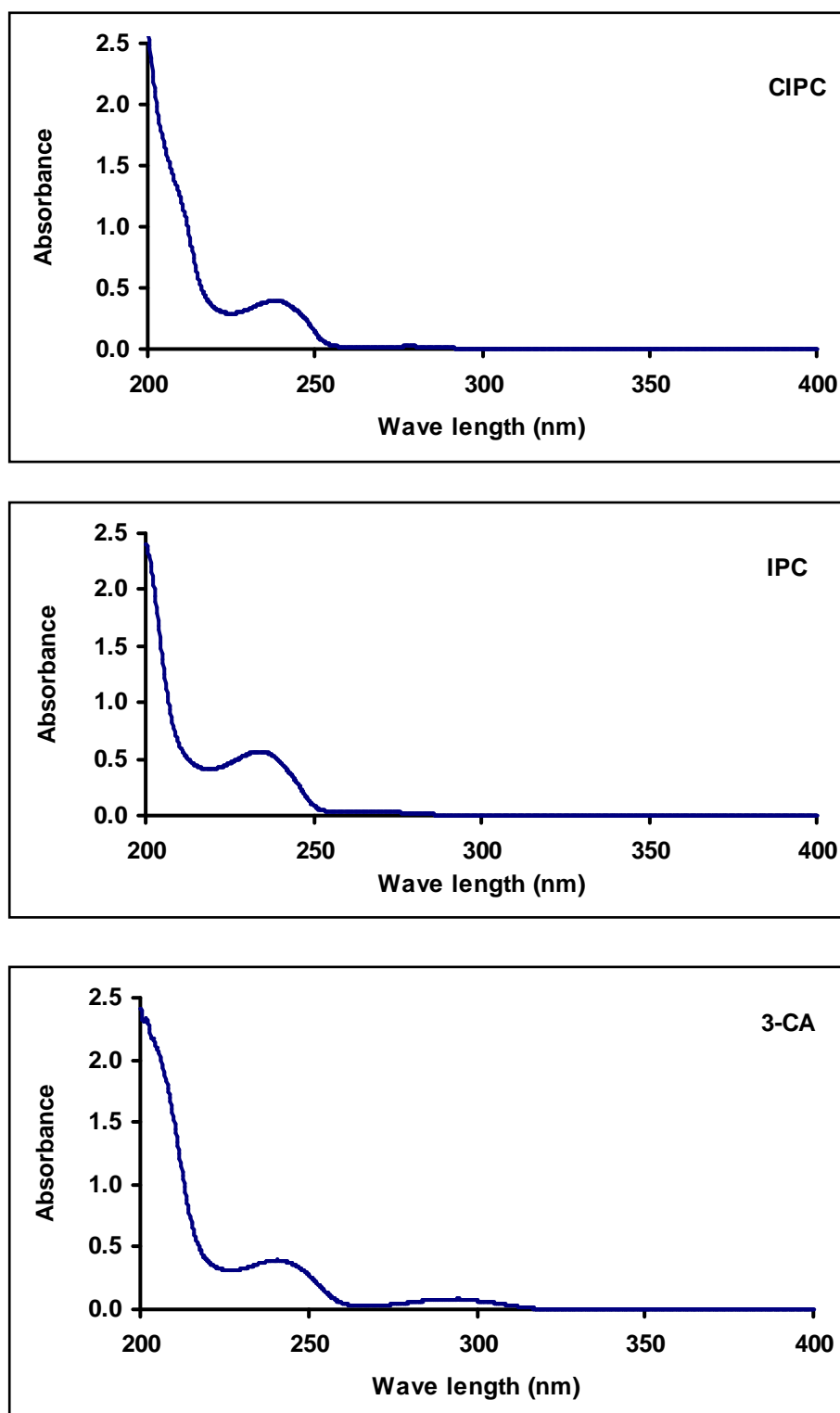
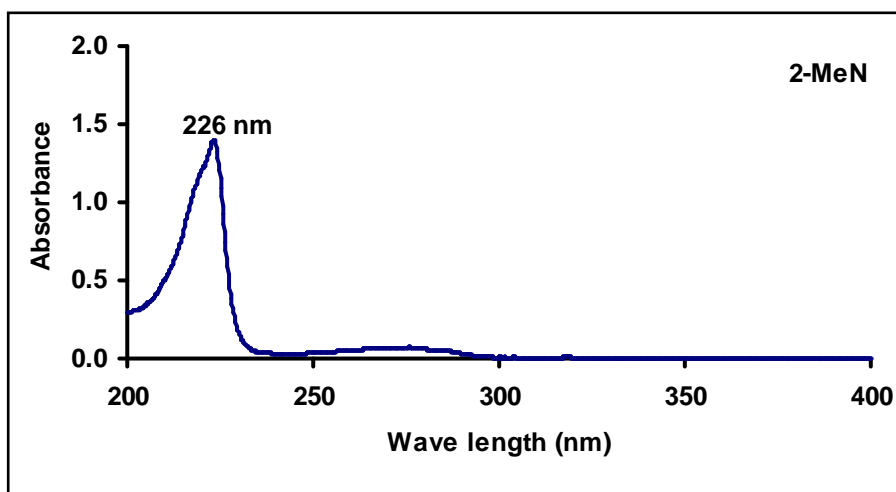
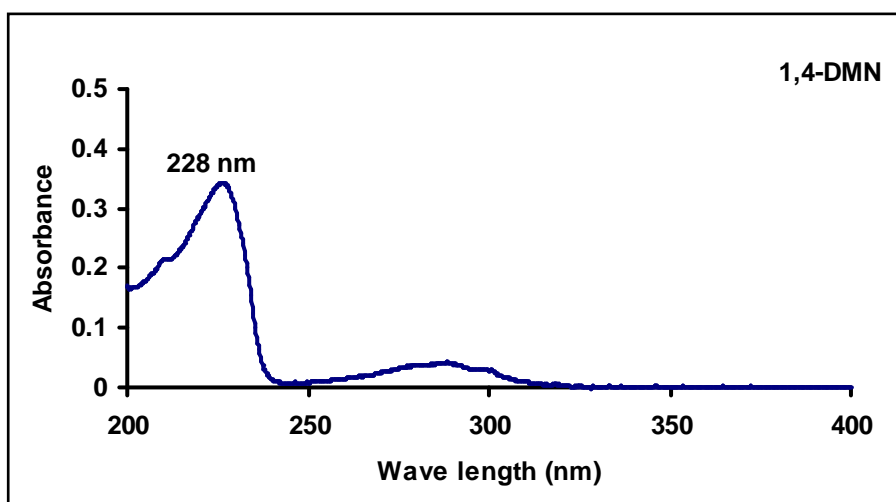
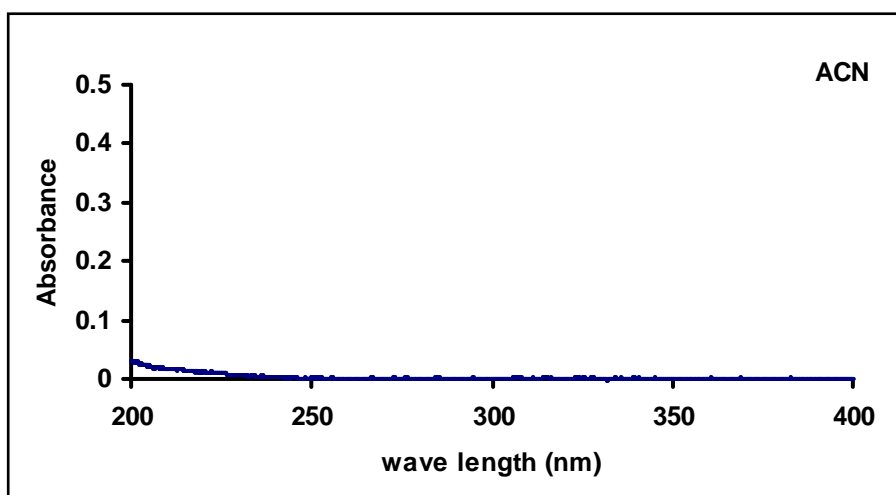


Figure 2:1. UV spectra of methanol and studied compounds in methanol solutions.

In contrast, using solutions of these compounds prepared in acetonitrile solvent showed strong UV absorbance at the optimum wavelength as shown in Figure 2:2.



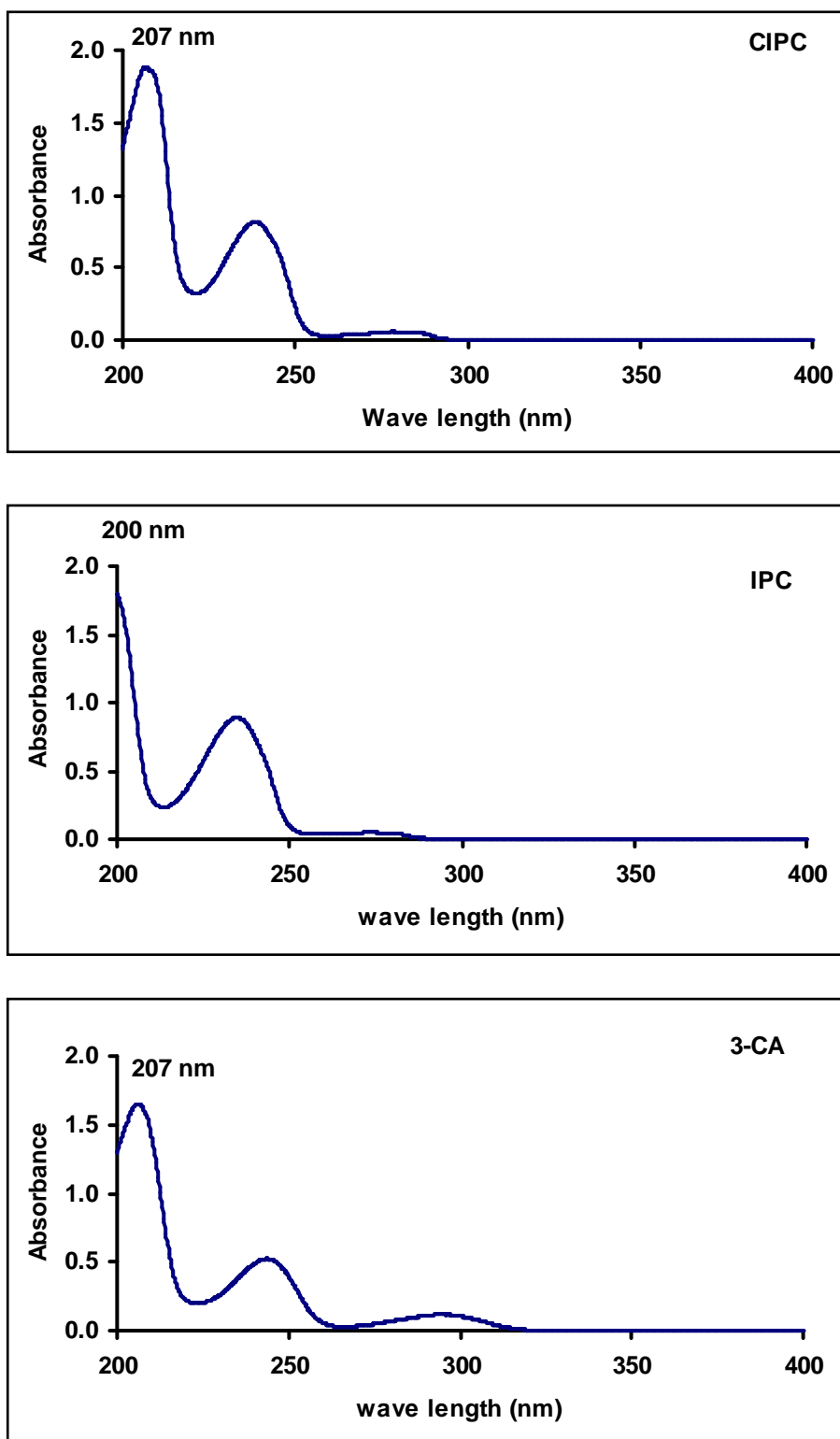


Figure 2:2. UV spectra of acetonitrile and studied compounds in acetonitrile solutions.

The optimum wavelength for 1,4-DMN at 228 nm was also confirmed by Beveridge (1979) and Oteef (2008). Therefore, this wavelength was selected and established in the separation method for the detector wavelength in the HPLC systems in this study. The optimum wavelength λ_{\max} of both CIPC and 3-CA was 207 nm which will be discussed later in Section 3.4.3.3.

Chapter 3: Development and validation of HPLC methods for the analysis of the potato sprout inhibitors 1,4-DMN and chlorpropham

3.1 Introduction

The fundamental aim of developing an analytical method is to separate and quantify the analyte in a mixture of compounds. A reversed phase HPLC (RP-HPLC) technique was selected for the determination of 1,4-dimethylnaphthalene (1,4-DMN), 2-methylnaphthalene (2-MeN) as internal standard, chlorpropham (CIPC) and its metabolite 3-chloroaniline (3-CA) using an internal standard of propham (IPC). Separation of the intended compounds is the first step of method development. To achieve the best separation with good resolution, the chromatographic conditions should be optimised selecting the specific detection wavelength and choosing an appropriate HPLC column, column temperature and mobile phase composition.

The type of organic solvent used for preparation of the mobile phase and standard solutions has a major role in RP-HPLC. The most commonly used solvents are acetonitrile, methanol and tetrahydrofuran. This project began using acetonitrile as the main solvent for developing a method for 1,4-DMN and its application in environmental samples. Because of the global shortage of acetonitrile of between 2008 and 2009, it was necessary to find an alternative solvent to acetonitrile, to continue this project. Methanol was selected as a potential substitute due to its similar separation characteristics to acetonitrile.

Commonly, the UV detectors used for HPLC are single wavelength detectors for quantitative analysis. Whilst, for more qualitative and quantitative information about the sample, diode-array detectors (DAD) may be employed to measure the absorbance at multi-wavelengths simultaneously.

Validating the HPLC method is crucial to prove the acceptability of the method and suitability for its intended purpose. In order to develop and validate a method, many specifications are required. Generally, development methods for regulatory submission should be based on studies of specificity, accuracy, precision, linearity, range, robustness, limit of detection and limit of quantification (Green, 1996).

Adding an internal standard to the calibration method is a good approach used to compensate for losses during sample preparation and instrumental measurement.

The work reported in this chapter describes the development of isocratic HPLC–UV methods for the analysis of the potato sprout inhibitors 1,4-DMN and chlorpropham and its degradation product 3-CA. To achieve an effective analytical method with efficient separation and high resolution, two major factors were investigated; column selection and the optimisation of the mobile phase composition. These RP-HPLC methods were validated for four major parameters including repeatability or intra-day precision, linearity, the limit of detection (LOD) and the limit of quantification (LOQ).

3.2 Method development and validation of 1,4-DMN and 2-MeN using different HPLC systems and acetonitrile as the eluent

3.2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are considered to be group of environmental contaminants that can have serious health effects, as many of this class of compounds are known to be carcinogenic (Jiang et al., 2011; Chen et al., 2005; Ruchirawat et al., 2010). Numerous applications of HPLC in the separation and quantification of PAHs in environmental and biological samples have been reported (Garcia-Falcon et al., 2004; Lu et al., 2011; Ren et al., 2010; Kicinski et al., 1989). One of the applications used involved HPLC coupled with ultraviolet absorption spectroscopy (UV) for the separation and identification of a series of PAHs (Xie et al., 1999). The analysis was performed on an ODS column using methanol–hexane (80:20) as mobile phase at flow rate of 1.0 mL/min and the UV detection was in the region 210 – 350 nm.

As mentioned previously, the scientific literature for the determination of 1,4-DMN is very limited. Very few numbers of analytical methods found were based on RP-HPLC. O'Hagan (1991) developed an analytical method for the analysis of 1,4-DMN residue in potato extract using stationary phased of C₁₈ octadecylsilane packed column and a mobile phase of methanol/water (70/30) mixed with 1 mL acetic acid, the retention time was 5 minutes at a flow rate of 2.0 mL/min and UV detection at 230 nm.

Recently, Oteef (2008) optimised an HPLC-UV separation method for the analysis of 1,4-DMN by studying the behaviour of a mixture of seven isomers of dimethylnaphthalene and other related compounds under different chromatographic conditions. The best separation was achieved using a Supleclo Supelcosil ODS-2 (C₁₈) column at a temperature of 12 °C with 40% (v/v) acetonitrile as a mobile phase at a flow rate of 1.5 mL/min and a chromatographic run time of 75 minutes. The method provided a good separation for most components in the mixture, in addition, good linearity and precision were obtained through method validation.

The main objective of the work in this section was to validate an HPLC separation method for the analysis of 1,4-dimethylnaphthalene (1,4-DMN) and 2-methylnaphthalene (2-MeN) (as an internal standard) employing three HPLC systems. A further objective was to

compare the sensitivity of a Hitachi diode array detector (DAD) and a SpectraSERIES UV100 single wavelength detector for the determination of these compounds. This initial step was important to select the best HPLC system optimising the chromatographic conditions prior to determination of 1,4-DMN in potato and environmental samples. The analytical method was validated according to international conference on harmonisation (ICH) guidance for validation of analytical procedure (ICH, 1994) by examining the precision of the HPLC instruments used in this study, validation of the linearity of the calibration curve and calculating the limit of detection (LOD) and the limit of quantification (LOQ).

3.2.2 Materials and methods

3.2.2.1 Materials and standards

See Sections 2.1.1 and 2.1.3 for preparation of the standard solutions (1,4-DMN and 2-MeN in ACN) and the mobile phase (from ACN and water) respectively.

3.2.2.2 HPLC systems

Three HPLC systems were used during this work; the brief details of these systems are summarised below:

- Hitachi (autosampler) system: an autosampler Merck Hitachi L-7200 and Merck Hitachi L-7100 pump were coupled to a Merck Hitachi L-4500 diode array detector (DAD), the output was recorded by Merck Hitachi L-7000 software version 4.1.
- SpectraSERIES UV100 (manual) system: the manual injector was a Rheodyne model 7125 and the pump used was a Cecil 1100 Series, these were connected with the thermo separation products SpectraSERIES UV100 detector and Dionex peaknet software.
- SpectraSERIES UV100 (autosampler) system: an autosampler Merck Hitachi L-7200 and Merck Hitachi L-7100 pump were coupled to a thermo separation products SpectraSERIES UV100 detector and Dionex peaknet software.

3.2.2.3 Chromatographic conditions

Separation was performed on the three HPLC systems under the same conditions using a Jones chromatography column (Hypersil ODS 5 μm , 250 mm x 4.6 mm) at ambient

temperature. The mobile phase consisted of 70% acetonitrile and 30% water at a flow rate of 1.5 mL/min and a run time of 10 minutes. The injection volume of the sample was 20 μ L and the detection was set at a wavelength of 228 nm.

3.2.2.4 Assessment of the precision for HPLC systems

The precision of the three HPLC systems was evaluated following repeated injections ($n = 10$) of 1 μ g/mL mixture of 1,4-DMN and 2-MeN by calculating the relative standard deviation (RSD%) (see Section 2.1.4.1).

3.2.2.5 Linearity of Calibration Curve

Two sets of mixed 1,4-DMN and 2-MeN standards were prepared. The first set of standards consisted of the following concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 μ g/mL. The second set of standards consisted of lower concentrations than the first set of 0.02, 0.04, 0.06, 0.08 and 0.10 μ g/mL. The different sets of standards were injected as duplicates into the three HPLC systems (see Section 2.1.4.2).

3.2.2.6 Limit of detection and quantification

Two procedures were applied to calculate the lowest concentration of detection (LOD) and lower limit of quantification (LOQ) of 1,4-DMN and 2-MeN. The first procedure consisted of repeated injections of two single solutions of 0.1 and 0.01 μ g/mL. Each solution was injected ten times into the three HPLC systems. The second approach derived the LOD and LOQ values statistically from the regression line of the lower range of the concentrations in the calibration curve (0.02 – 0.1 μ g/mL) as described in Section 2.1.4.3.

3.2.3 Results and discussion

3.2.3.1 Chromatographic conditions

The separation process as a preliminary step plays a critical role in quantitative analysis and method development. The main aim of this proposed work was to choose a simple method achieving good separation of 1,4-DMN and its internal standard 2-MeN employing three HPLC systems under the same chromatographic conditions. In this study, 2-MeN was used as an appropriate internal standard to mimic the variation of any loss of 1,4-DMN during the sample preparation or instrumental analysis. It was also selected by Oteef (2008) for the resemblance of its behaviour to 1,4-DMN in the extraction and chromatographic separation procedures after comparing it with a number of other isomers

and related compounds (such as 2-ethylnaphthalene, 1-ethylnaphthalene and n-butylbenzene).

The chromatographic conditions chosen in this study achieved good separation with high resolution between the closely eluted peak 2-MeN and 1,4-DMN peak at a short retention time of approximately between 4.5 – 5.5 and 5.5 – 7.0 minutes respectively, as shown in Figure 3:1.

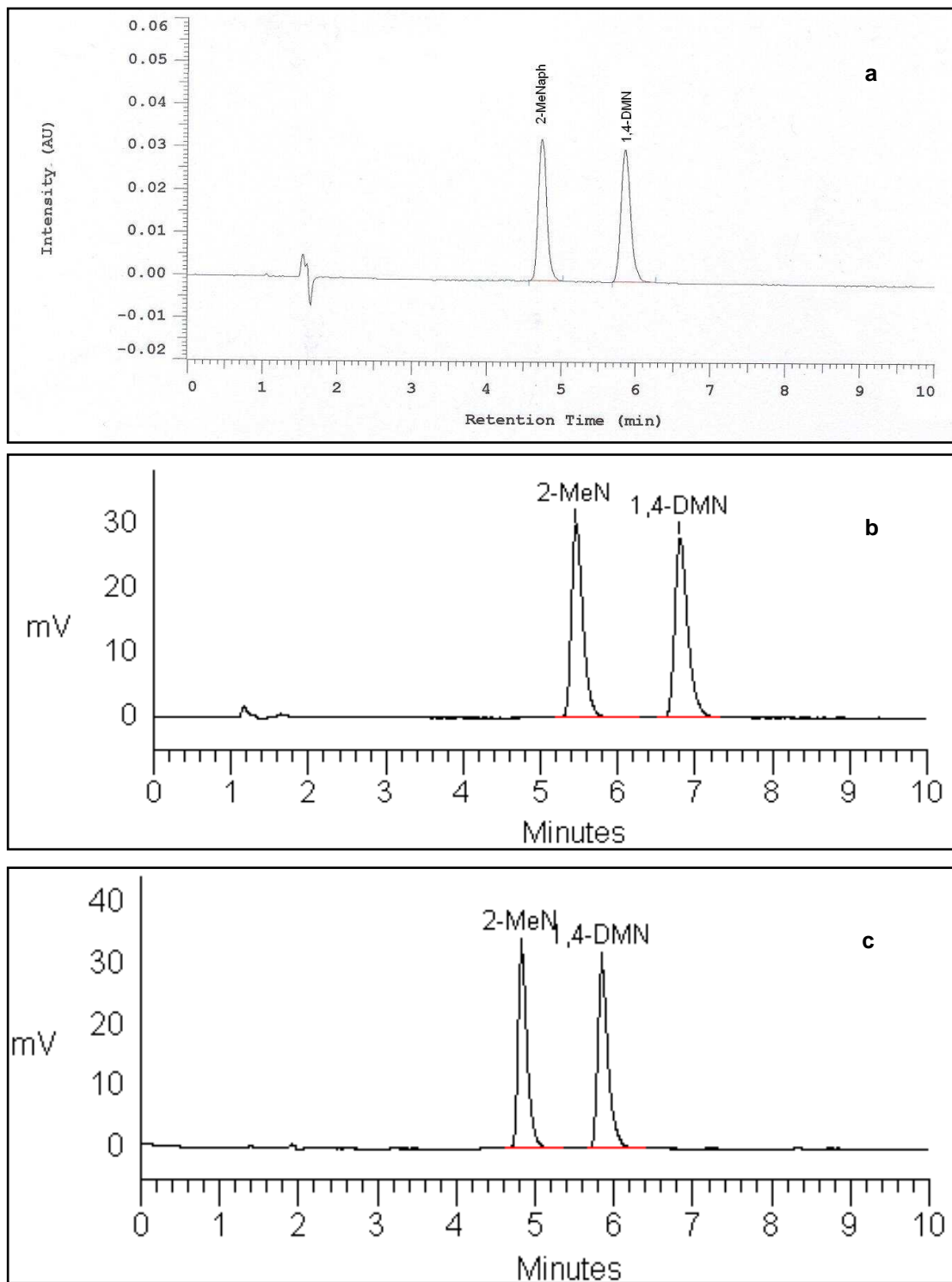


Figure 3:1. Chromatograms of 1 µg/mL mixture of 1,4-DMN and 2-MeN of three HPLC systems: a- Hitachi DAD-autosampler, b- SpectraSERIES UV100-manual injector and c- SpectraSERIES UV100-autosampler.

This slight variability of the retention time between chromatographic systems can be due to various factors regarding the different specification of each HPLC system, column temperature and length of tubing between the injector and column. In particular, the column aging and the prolonged usage of the Jones chromatography column could also cause the drifting in the retention time.

Ultimately, some modifications are required for the chromatographic conditions. For example, the column temperature during this experiment was ambient and not controlled and in order to overcome the temperature effect on the retention time, a column oven is recommended.

3.2.3.2 Assessment of the precision for the HPLC systems

Precision is important to achieve consistent quantitative data. A peak area is preferred for precision calculation over peak height due to the variability of using the peak height, which is effected by some parameters such as the column temperature and the flow rate of the mobile phase (Snyder et al., 2010; Bakalyar and Henry, 1976).

The precision results on the Hitachi- autosampler HPLC system showed little variation in the peak area between the chromatographic runs for both 1,4-DMN and 2-MeN when compared with high variability of the SpectraSERIES UV100-manual injector system as shown as the RSD% of peak areas in Table 3:1.

Table 3:1. RSD% values estimated of drifting the peak area on two HPLC systems.

HPLC system	Injector	RSD% of peak areas (n = 10)	
		2-MeN	1,4-DMN
Hitachi DAD	Autosampler	0.91	0.56
SpectraSERIES UV100	Manual	2.90	3.10

The precision of the Hitachi DAD-autosampler system was better than the SpectraSERIES UV100-manual injector system, possibly due to the use of manual injector. However, the precision of the manual injector can be increased by calculating the ratio of the peak area of 1,4-DMN to 2-MeN for ten replicates to give an RSD% of 0.6. When plotting the peak areas of both compounds against the injection number, the SpectraSERIES UV100-manual injector system showed high drifting as well as variability, as shown in Figure 3:2.

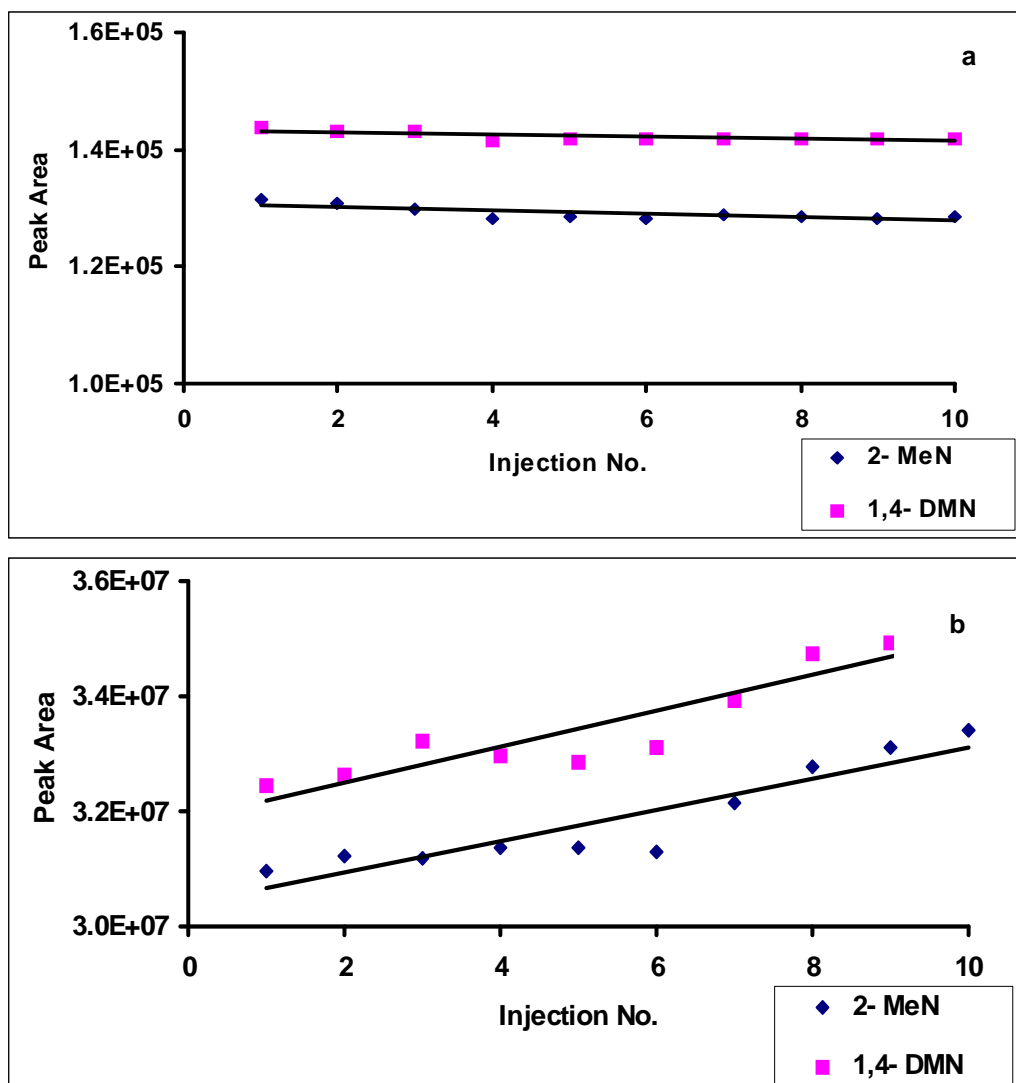


Figure 3:2. Drifting the peak area of 2-MeN and 1,4-DMN during the day on: a- Hitachi DAD-autosampler HPLC system and b- SpectraSERIES UV100-manual injector HPLC system.

Numerous reasons can contribute to the fluctuation in the peak area. Mainly, drifting was caused by systematic variability, which changed considerably with time and influenced the precision of the measurement. Variations in room temperature can cause real problems for precision measurements by influencing the column temperature and subsequently causing drift in the retention time and peak area. The influence of column temperature in LC is a significant parameter in method development and normally, ambient temperature is used. Lowering the temperature increases the mobile phase viscosity, which in turn increases the total analysis time and column pressure. Therefore, the resolution, selectivity, analysis time and column pressure are affected by changing the column temperature (Yoshida and Majors, 2006).

The influence of temperature on the precision of retention measurements has been investigated for both reversed phase and normal phase systems (Gilpin and Sisco, 1980). It was shown that the precision of reversed phase systems was good and the largest deviations in retention as function of temperature occur when the mobile phase was totally aqueous. Whilst for normal phase which is more likely to be affected by temperature fluctuations, the largest degree of error in solute retention was observed when the chromatographic system included a polar stationary phase with a polar mobile phase modifier. Thus, these results indicated the importance of the temperature control to determine the level of precision in measuring solute retention.

Scott and Reese (1977) studied the effect of minimum difference in temperature and composition of the mobile phase on the precision of chromatographic measurements. They recommended that prior to entering the column, the mobile phase should be at a fixed temperature and a constant density to keep the volume flow rate inside the column constant. Subsequently, the pump has to carry a constant mass flow rate to the column. Additionally, to achieve the required precision measurement, the ambient temperature of the apparatus room should be controlled and maintained (Scott and Reese, 1977).

In the present study, the major focus was to improve the precision of the peak area by stabilising the column temperature. Some temperature variation of the mobile phase and standard solution had an effect on the column temperature. Therefore, the temperature of the chromatographic system needs to be fixed at (or slightly above) ambient temperature, which is commonly between 20 °C and 25 °C. Stabilising the temperature was controlled by insulating the mobile phase and injecting solutions by placing the reservoir of the mobile phase in a polystyrene box. In addition, the mobile phase was prepared the day before it was required for analysis and kept overnight at a fixed room temperature of 20 °C. Moreover, the standard solutions were taken out of the refrigerator and warmed to room temperature prior to injection. Furthermore, in order to overcome the temperature effect, controlling the column temperature is required using a column oven coupled with cooling devices (was not available at this part of study) to obtain stable chromatographic conditions.

A big improvement in the precision was achieved after stabilising the temperature and re-running the ten replicate injections of the standard solution of 1 µg/mL of 1,4-DMN and 2-MeN on the three HPLC systems as shown in Table 3:2.

Table 3:2. RSD% values of the peak area estimated after temperature stability on three HPLC systems.

HPLC system	Injector	RSD% of peak areas (n = 10)	
		2-MeN	1,4-DMN
Hitachi DAD	Autosampler	0.07	0.11
SpectraSERIES UV100	Manual	0.91	0.82
SpectraSERIES UV100	Autosampler	0.80	0.16

When plotting the peak area against the injection number, little drifting of either compound was found on the three HPLC systems as shown in Figure 3:3.

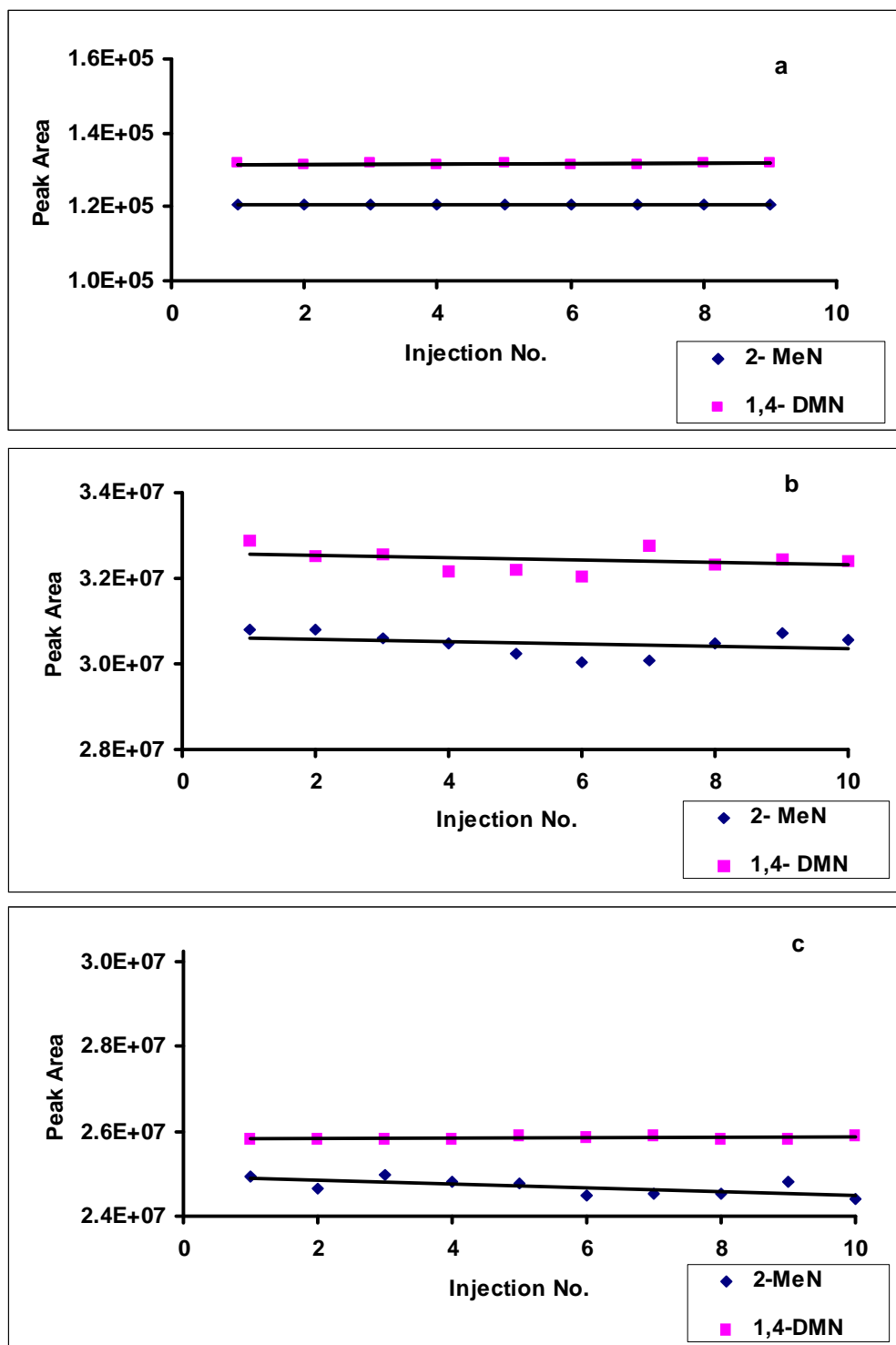


Figure 3:3. The effect of stability of temperature on the peak area for both 1,4-DMN and 2-MeN on: a- Hitachi DAD-autosampler HPLC system, b- SpectraSERIES UV100-manual injector HPLC system and c- SpectraSERIES UV100-autosampler.

The three HPLC systems achieved good precision for both compounds (2-MeN and 1,4-DMN) as shown by RSD% values of the peak area of less than 1% . The value of RSD% is suggested to be $\leq 1\%$ as an appropriate precision criterion for repetitive injections to assess the precision of the instrument in analytical method validation. An RSD% of $\leq 5\%$ will be an acceptable instrumental precision for a method at low level concentrations close to the limit of detection (Green, 1996). In addition, an RSD% of 1% or less is acceptable for the

precision criteria in particular of the autosampler performance for at least six replicates (Hall and Dolan, 2002; Shabir et al., 2007). Therefore, in this study the criterion for method precision was selected to be a relative standard deviation of less than 1%.

In this study, the low RSD% values indicated satisfactory repeatability of the HPLC method. However, the precision of the analytical method was better on the autosampler injector systems (Hitachi DAD and SpectraSERIES UV100 systems) than SpectraSERIES UV100-manual injector. These results illustrate the ability of this method and the efficiency of these HPLC systems to be applied to the routine analysis of 1,4-DMN residues in potatoes and other environmental samples.

3.2.3.3 Linearity of Calibration Curve

Assessment of the linearity of the calibration curve is recommended to prove the acceptability of any analytical method (Green, 1996). Generally, to verify the linearity, five concentration levels of standard solutions are required to construct the regression line of the calibration curve. In this study, a linearity test was performed by plotting the calibration curve between the standard concentration and the detector response. The linearity can be examined through the correlation coefficient (r) which is often used as linearity measure of the calibration curve. In chemical correlation analysis, coefficient of determination (R^2) is the more exact term used (Exner and Zvara, 1999). In this study, the linearity criterion was chosen using R^2 of the regression line, which is suggested to be 0.990 or more. On this basis, the results illustrate a good linearity between the peak area and the concentrations of the standard solutions of each of 1,4-DMN and 2-MeN on all three HPLC systems as shown in the following figures:

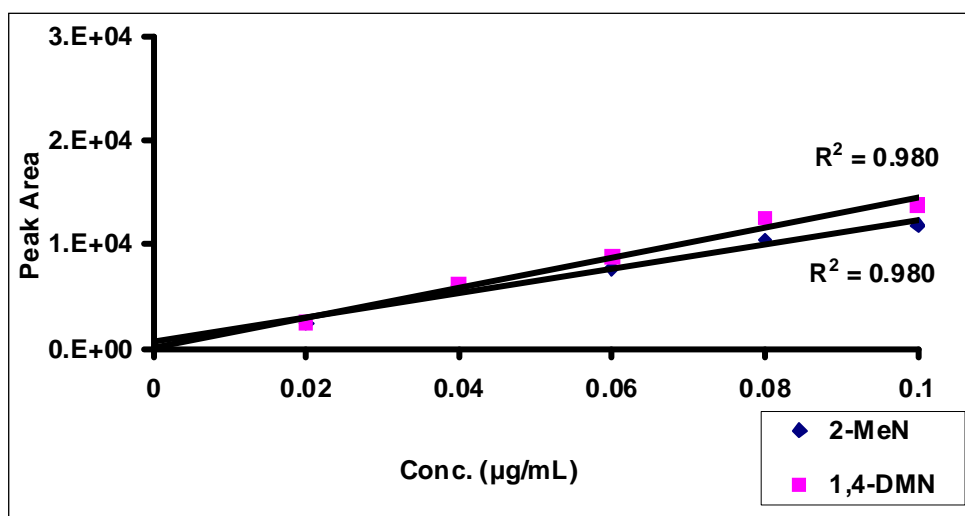


Figure 3:4. Calibration graph for 2-MeN and 1,4-DMN at range 0.02 – 0.1 µg/mL on the Hitachi DAD-autosampler HPLC system.

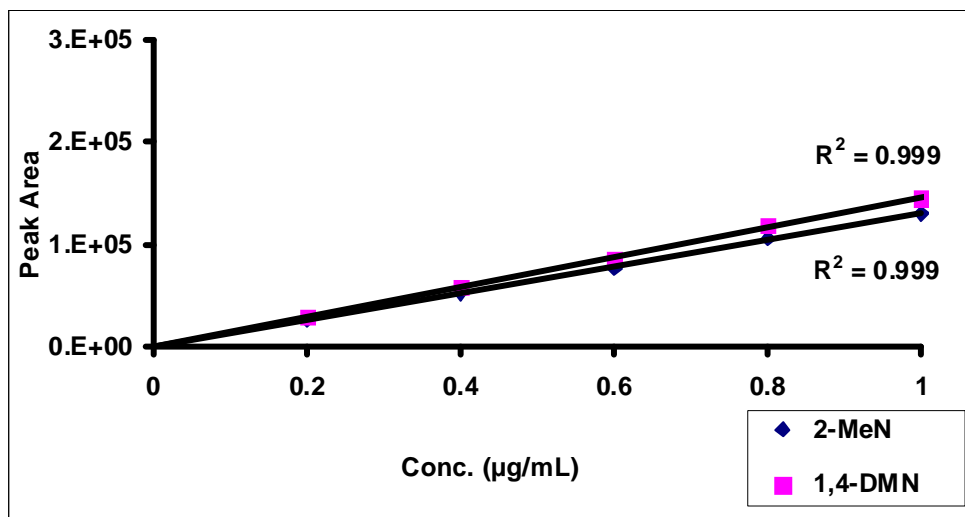


Figure 3:5. Calibration graph for 2-MeN and 1,4-DMN at range 0.2 – 1.0 µg/mL on the Hitachi DAD-autosampler HPLC system.

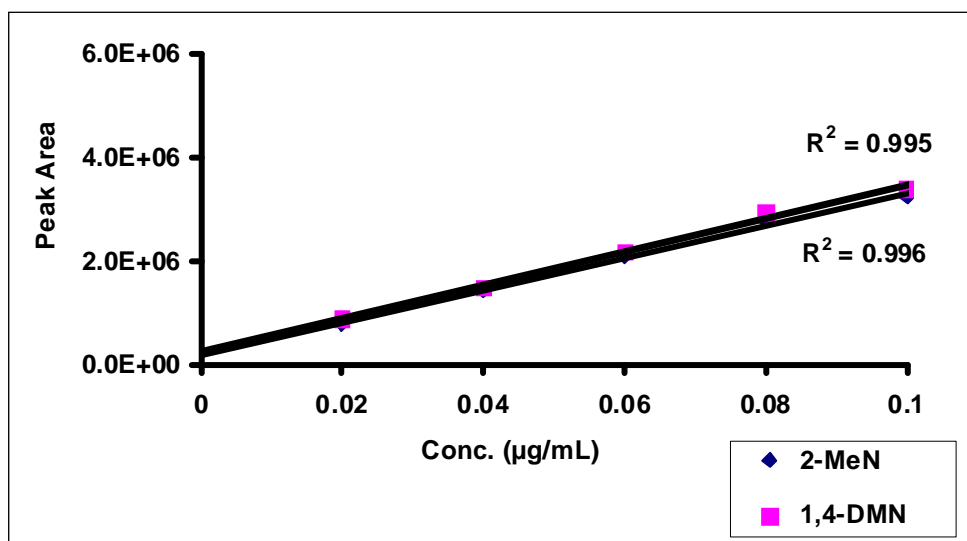


Figure 3:6. Calibration graph for 2-MeN and 1,4-DMN at range 0.02 – 0.1 µg/mL on the SpectraSERIES UV100-manual injector HPLC system.

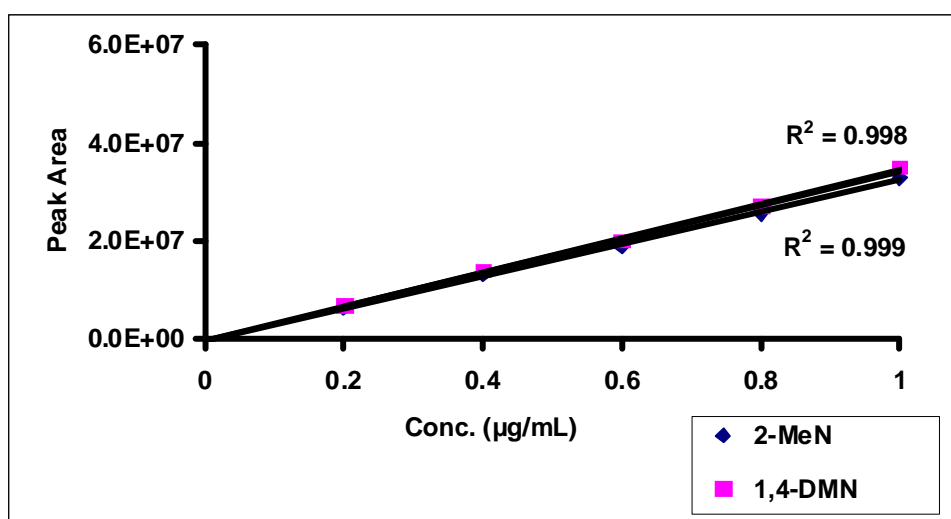


Figure 3:7. Calibration graph for 2-MeN and 1,4-DMN at range 0.2 – 1.0 µg/mL on the SpectraSERIES UV100-manual injector HPLC system.

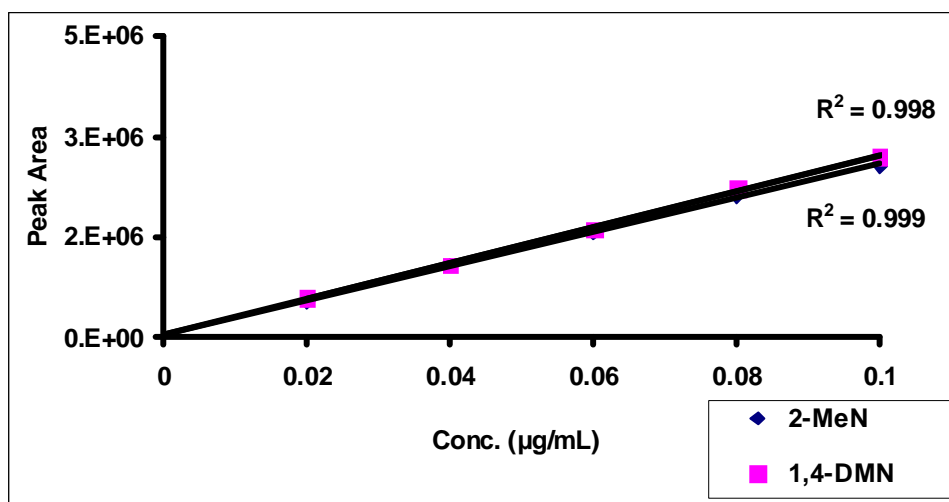


Figure 3:8. Calibration graph for 2-MeN and 1,4-DMN at range 0.02 – 0.1 µg/mL on the SpectraSERIES UV100-autosampler HPLC system.

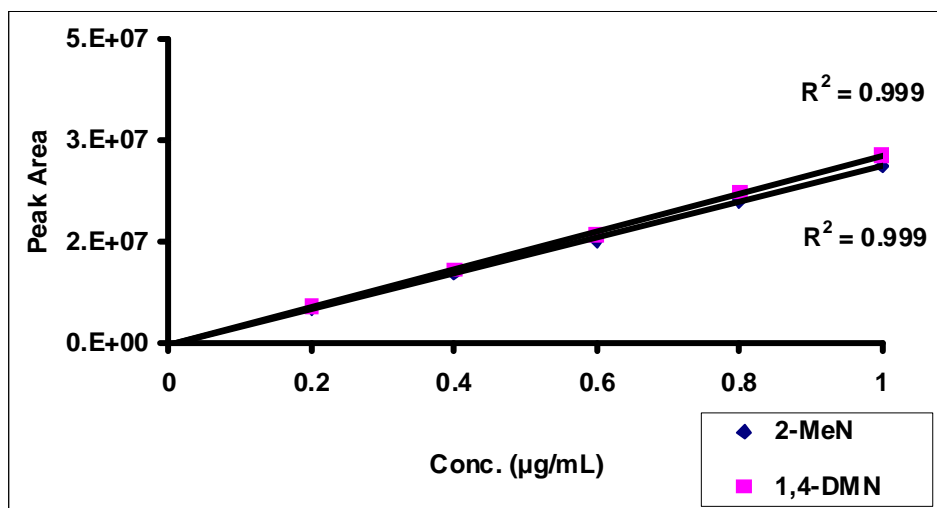


Figure 3:9. Calibration graph for 2-MeN and 1,4-DMN at range 0.2 – 1.0 µg/mL on the SpectraSERIES UV100-autosampler HPLC system.

3.2.3.4 Limit of detection and quantification

The LOD and LOQ were calculated using two approaches, firstly by repeated injections ($n = 10$) of each of the two solutions of 0.1 and 0.01 $\mu\text{g/mL}$ mixture of 1,4-DMN and 2-MeN just above the expected LOQ approximately ($5 * \text{LOQ}$) for the three HPLC systems as shown in Tables 3:3 and Table 3:4.

Table 3:3. LOD and LOQ values for repeatability injection of 0.1 $\mu\text{g/mL}$ mixture of 2-MeN and 1,4-DMN on each HPLC system.

HPLC system	Injector	LOD ($\mu\text{g/mL}$)		LOQ ($\mu\text{g/mL}$)	
		2-MeN	1,4-DMN	2-MeN	1,4-DMN
Hitachi DAD	Autosampler	0.003	0.003	0.011	0.008
SpectraSERIES UV100	Manual	0.007	0.006	0.025	0.018
SpectraSERIES UV100	Autosampler	0.003	0.002	0.009	0.007

Table 3:4. LOD and LOQ values for repeatability injection of 0.01 $\mu\text{g/mL}$ mixture of 2-MeN and 1,4-DMN on each HPLC system.

HPLC system	Injector	LOD ($\mu\text{g/mL}$)		LOQ ($\mu\text{g/mL}$)	
		2-MeN	1,4-DMN	2-MeN	1,4-DMN
Hitachi DAD	Autosampler	0.003	0.004	0.010	0.015
SpectraSERIES UV100	Manual	0.001	0.001	0.002	0.004
SpectraSERIES UV100	Autosampler	0.001	0.001	0.002	0.002

The second approach estimated the LOD and LOQ statistically from the calibration curve at the lower range of concentrations 0.02 – 0.1 $\mu\text{g/mL}$ on the three HPLC systems as shown in Table 3:5.

Table 3:5. LOD and LOQ values on each HPLC system based on the statistical data for the calibration curve in the range 0.02 – 0.1 $\mu\text{g/mL}$ for 2-MeN and 1,4-DMN.

HPLC system	Injector	LOD ($\mu\text{g/mL}$)		LOQ ($\mu\text{g/mL}$)	
		2-MeN	1,4-DMN	2-MeN	1,4-DMN
Hitachi DAD	Autosampler	0.014	0.017	0.046	0.057
SpectraSERIES UV100	Manual	0.007	0.008	0.024	0.028
SpectraSERIES UV100	Autosampler	0.004	0.004	0.013	0.013

To compare the reliability of the presented results of LOD and LOQ for the two approaches, these values undoubtedly are different because the calculation of a standard deviation for each approach is different. The values for the repeated injections approach are lower and more realistic in practical use, whereas the graphical method shows higher values as the assumption of a constant standard deviation at all concentrations is probably not true. However, all three systems offered acceptable LOD and LOQ values despite the difference in the specification of the Hitachi DAD and SpectraSERIES UV100 detectors.

Selecting a suitable detector depends on the intended purpose of the method and the detection limit of the analyte that is being determined. In the present work, two detectors were operated; diode array and SpectraSERIES UV100 detectors. DAD-autosampler HPLC system presented LOD and LOQ values for 1,4-DMN higher than SpectraSERIES UV100 detector-autosampler system. The diode array detector can be used to monitor multiwavelength and provide an entire spectra of all the peaks during the chromatogram run (Remcho et al., 1992). It can also monitor the peak purity that will be required for further investigation in this study.

3.2.4 Conclusion

Three HPLC systems were operated with the aim of validating separation methods for the analysis of 1,4-dimethylnaphthalene and its internal standard 2- methylnaphthalene and choosing the best system. A successful validation verified the capability of each system to offer reliable chromatography. All the chromatograms demonstrated that each system could achieve excellent separation under the same chromatographic conditions. The analysis was performed isocratically on a Jones-ODS column under chromatographic conditions of 70:30 of acetonitrile: water mobile phase at a flow rate of 1.5 mL/min, 20 μ L injection volume at a run time of 10 minutes and a detection wavelength of 228 nm.

The precision of the analytical method on SpectraSERIES UV100-manual system indicated high drifting in the peak area between the replicate injections when compared with the Hitachi-autosampler system. Stabilisation of the temperature of the mobile phase and injected solutions achieved a reasonable precision (RSD% < 1%) for all three HPLC systems in particular the autosampler systems were better than manual injector.

All systems showed a good linearity of the calibration curves at two ranges of the concentration through the obtained R^2 for the regression line of each 1,4-DMN and 2-MeN. According to different views in the literature of linearity criteria, a coefficient of

determination (R^2) of the regression line of 0.990 or more will be acceptable for good linearity for analytical method validation in this study.

The LOD and the LOQ were estimated based on two approaches of measuring the standard deviation and the values were found to be variable between the approaches. Based on statistical data of the regression line, this approach resulted in higher values for both the LOD and the LOQ of 1,4-DMN. In contrast, lower LOD and LOQ values were obtained with repeated injection and this approach offered lower values with all three systems.

Ultimately, this comparison between the validation of the three HPLC systems highlighted that the SpectraSERIES UV100-autosampler system offered the best chromatographic results suited to the analysis of 1,4-DMN in potatoes and environmental samples. For this reason, this system was proposed as the final system to use for the remainder of this research. However, some essential modifications were required to achieve the best chromatographic conditions on this system such as the column oven and cooling device to overcome any temperature effects.

SpectraSERIES UV100-autosampler HPLC system under the same chromatographic conditions were used for the quantitative analysis and adsorption onto laboratory ware of 1,4-DMN and its internal standard 2-MeN in aqueous solution. However, this work was suspended due to the global shortage of acetonitrile (See Section 4.2).

3.3 Development and validation of an HPLC method for the analysis of 1,4-DMN and 2-MeN using methanol as an eluent

3.3.1 Introduction

The global economic downturn of 2008 – 2009 caused a shortage of acetonitrile, which is mainly obtained as a by-product in the production of acrylonitrile (Purdie et al., 2009). The global shortage of acetonitrile was attributed to the significant reduction and slowdown in industrial spending on acrylonitrile. Because of this reduction, the supply of acetonitrile was not expected to return to normal levels during this work. It was therefore important to look for an alternative solvent to acetonitrile.

Acetonitrile is the most commonly used solvent in reverse-phase HPLC separations for many reasons. It has excellent chromatographic properties due to its high polarity, low viscosity and good selectivity properties. In addition, it provides a low spectroscopic cut off (background absorbance < 0.05 AU) of 190 nm. Furthermore, acetonitrile has very good solubilising properties. Therefore, for chromatographic purposes, replacing the solvent will be very complicated.

The global shortage of acetonitrile affected the use of HPLC and compelled researchers in this field to find substitutes for acetonitrile. Some essential factors need to be taken into consideration during the selection of alternative solvents for HPLC, particularly in terms of the chemical and physical properties of the solvent that have consequent effects on the chromatographic process such as separation, detection limits and analytical reproducibility. Replacing solvents with alternatives can influence some chromatographic factors related to retention time, peak shape, efficiency, symmetry, resolution and selectivity.

A typical parameter for solvent selection is based on the UV cut off which should not be higher than the working wavelength used for an analysis to avoid generating high background absorbance. Methanol was considered as a potential substitute and gives a similar separation to acetonitrile. However, the UV cut off for methanol is 205 nm whereas for acetonitrile is 190 nm. This may be a significant consideration when replacing the solvent as part of the method development. Methanol is less expensive than acetonitrile. However, it is a weaker solvent, thus a higher percentage of methanol in the mobile phase is essential for elution. Commercially, the purity of HPLC grade methanol appears better

when compared with acetonitrile; although the drifting of the gradient baseline with methanol is higher than with acetonitrile due to the high range of absorbance of methanol 190 – 260 nm (Williams, 2004). It is also relevant to point out that the selectivity varies between acetonitrile and methanol due to the different solvation properties of each and the ability of methanol to effect the hydrogen bonding between the analytes and polar groups on the column.

To overcome this problem of acetonitrile shortage, it was essential to continue investigating to develop and validate a routine method of HPLC to ultimately be able to analyse the sprout inhibitor 1,4-DMN using an alternative eluent. For this purpose, methanol was selected as a substitute solvent.

3.3.2 Materials and methods

3.3.2.1 Materials and standards

See Section 2.1.1 for the preparation of standard solutions of 1,4-DMN and 2-MeN in methanol and Section 2.1.3 for the preparation of a mobile phase from methanol and water.

3.3.2.2 Equipment

The HPLC system described in Section 2.1.2 was used with exception using column oven and cooling device at this part. The Jones Hypersil ODS column was also used.

3.3.2.3 Optimising the separation of 1,4-DMN and 2-MeN using different strengths of the mobile phase

The chromatographic conditions for separation of 1,4-DMN and 2-MeN were set using a 20 µL injection volume and UV detection at a wavelength of 228 nm. An isocratic method was employed using different concentrations of the mobile phase (50%, 60%, 70%, 80%, 85% and 90% (v/v)) of methanol at a flow rate of 1.5 mL/min.

3.3.2.4 Determination of precision

The precision in terms of repeatability (intra-day precision) of the autosampler HPLC system was determined by ten replicate injections of each methanol standard solution of 1 and 10 µg/mL of mixed 1,4-DMN and 2-MeN.

3.3.2.5 Linearity of the calibration curve

Three sets at different concentration ranges 0.02 – 0.1, 0.2 – 1.0 and 2 – 10 µg/mL of mixed standards of 1,4-DMN and 2-MeN were prepared to assess the linearity of the calibration curve.

3.3.2.6 Limit of detection and quantification

The limit of detection and limit of quantification were estimated as explained in Sections 3.2.2.6 and 2.1.4.3.

3.3.3 Results and discussion

3.3.3.1 Optimising the separation of 1,4-DMN and 2-MeN using different strengths of the mobile phase

Methanol was selected for RP-HPLC analysis as the elution solvent for the studied compounds due to its water miscibility and eluting efficiency. The composition of the mobile phase is one of the most important parameters used to control HPLC retention and optimise the separation of eluted compounds. The effect of the strength of the mobile phase was investigated to describe retention changes of 1,4-DMN and its internal standard 2-MeN. This initial step of separation started on a Jones chromatography column (Hypersil ODS 5 µm, 250 mm x 4.6 mm) using different concentrations of methanol in water v/v% (50%, 60%, 70%, 80%, 85% and 90%) as the mobile phase at a flow rate of 1.5 mL/min. The increase in the MeOH strength led to a decrease in retention factors for both 1,4-DMN and 2-MeN that can be explained by the reversed phased HPLC caused by the hydrophobic interaction between the solute and the adsorbent, which is reduced when increasing the amount of organic mobile phase solvent (Ching et al., 2000).

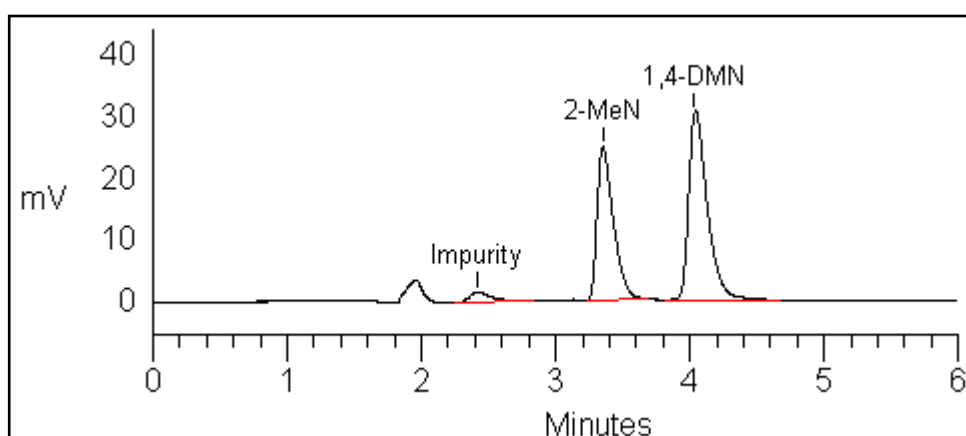
A good RP-HPLC separation of 1,4-DMN and 2-MeN with good resolution was obtained using all the strengths of the mobile phase but with different run times (see Table 3:6).

Table 3:6. Different concentrations of the mobile phase (methanol%) to separate 1,4-DMN and 2-MeN at different retention times.

Mobile phase concentration (v/v methanol %)	Run time (minute)	Retention time (minute)	
		2-MeN	1,4-DMN
50	100	64	NP*
60	50	27	45
70	20	10	15
80	10	5.5	7.5
85	10	4.0	5.0
90	6	3.5	4.0

NP* no peak

However, a shorter run time with very good resolution was accomplished after 6 minutes by using 90% methanol as shown in Figure 3:10.

**Figure 3:10. Chromatogram showing the separation of 1,4-DMN and 2-MeN on Jones (Hypersil ODS) column using 90% concentration of the mobile phase (methanol%) and ambient temperature.**

This typical chromatogram shows that the resolved peaks tailed or became asymmetrical with a broader shape close to the baseline. Broadening and tailing of the peak principally make it difficultly to detect exactly the end of the peak therefore reducing the quality of the chromatogram. Consequently, this can affect the accuracy and precision of the system. Most often, the broad peaks are noticeable in an isocratic separation. This can be accounted for either by the adsorption of impurities in the column or by the deterioration of the column (particularly silica-based packing material). Generally, in all chromatography, longitudinal diffusion is responsible for increasing the bandwidth of the separating components. In isocratic elution chromatography, the components should be eluted before

the longitudinal diffusion becomes uncontrollable resulting in broad peaks (Williams, 2004).

Washing the column is an easy step that can dramatically improve the separation and peak shape of eluted peaks. Therefore, washing the column with 100% methanol for 30 minutes was undertaken. Reversing the column was also done during washing process to increase the exiting rate of the solubilised contaminants from the column because most of the strongly held contaminants are usually at the head of the column (Majors, 2003). However, the chromatograms showed no improvement in terms of their peak shape. Heavily used columns can usually be the cause of tailing peaks (Snyder et al., 1988). In particular, the column that was used in this separation (Jones Hypersil ODS) has been used for long term analysis. Thus, this column was replaced following an examination of available columns in an attempt to obtain a good peak shape. A Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Sphereclone) column solved the problem and produced peaks with little or no peak tailing as shown in Figure 3:11.

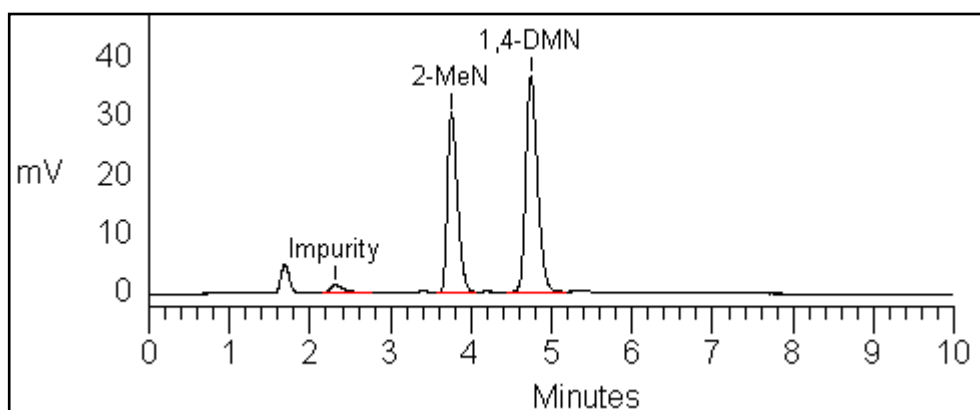


Figure 3:11. Chromatogram showing the separation of the eluted compounds on Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Sphereclone) at 90% methanol with a flow rate of 1.5 mL/min at ambient temperature.

To protect the analytical column from any contamination, a guard column was installed. Usually, the specifications of the cartridge of the HPLC guard column are the same as the packed material and also the same internal diameter as the analytical column with short length; the guard column should be discarded when it becomes contaminated. The column was at an ambient temperature of approximately 20 °C. In addition, the mobile phase was insulated against the temperature changes as discussed in Section 3.2.3.2 by placing the mobile phase reservoir in a polystyrene box. Furthermore, preparation of the mobile phase was performed a day in advance of the analysis and was stored along with standard solutions overnight at a fixed room temperature of 20 °C.

From the chromatogram in Figure 3:11, the final chromatographic conditions selected for this method can be summarised as the following:

- Column: Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Sphereclone)
- Guard column: Phenomenex[®] Security Guard[™]
- Detector: SpectraSERIES UV100
- Wavelength detection: 228 nm
- Mobile phase: 90% methanol: 10% water
- Flow rate: 1.5 mL/min
- Chromatographic run: 10 minutes.
- 1,4-DMN retention time: ~ 5 minutes.
- 2-MeN retention time: ~ 4 minutes.
- Injection volume: 20 μ L
- Column temperature: ambient ~ 20 °C

3.3.3.2 Determination of precision

Validation of the method was performed through examining the precision. The RSD% values for the peak area are presented in Table 3:7 and were found to be less than 1% and evidenced an excellent precision on the basis of precision criteria previously discussed (see Section 3.2.3.2).

Table 3:7. RSD% values for the peak area of 2-MeN and 1,4-DMN.

Concentration	RSD% (n = 10)	
	2-MeN	1,4-DMN
1 μ g/mL	0.9	0.9
10 μ g/mL	0.7	0.3

3.3.3.3 Linearity of the calibration curve

Linearity was evaluated through the regression line of the calibration curve. The linearity details of the coefficient of determination (R^2) of the calibration line of each compound at each level of concentration are as presented in Table 3:8.

Table 3:8. Coefficient of determination values of the calibration curve for 2-MeN and 1,4-DMN at different ranges of the concentration.

Compound	Conc. range (µg/mL)	Correlation of determination (R^2)
2-MeN	0.02 – 0.1	1.000
	0.2 – 1.0	1.000
	2.0 – 10	0.997
1,4-DMN	0.02 – 0.1	0.997
	0.2 – 1.0	1.000
	2.0 – 10	1.000

The results of the coefficient of determination were found to be better than 0.990 which is the level chosen for the linearity criteria for this study (see Section 3.2.3.3). A good linearity demonstrated that no significant deviation in the peak area response over the concentration of compounds at each level. These ranges of concentration can now be employed for the intended application of the test method.

3.3.3.4 Limit of detection and quantification

The LOD and LOQ were determined at low concentrations by two approaches of standard deviation measurement (replicate injections and the graphical method) (see Table 3:9). The values obtained by repeated injection of a 0.01 µg/mL standard showed a lower LOD and LOQ than that obtained from the standard deviation of the regression line as discussed in Section 3.2.3.4.

Table 3:9. LOD and LOQ values based on the repeatability injection (n = 10) of a 0.01 µg/mL mixture of 2-MeN and 1,4-DMN and the statistical data for the calibration curve in the range 0.02 – 0.1 µg/mL of the mixed standards.

Assessed Approach	LOD (µg/mL)		LOQ (µg/mL)	
	2-MeN	1,4-DMN	2-MeN	1,4-DMN
Injection repeatability (0.01 µg/mL)	0.0003	0.0001	0.0005	0.0009
Calibration curve (0.02 – 0.1 µg/mL)	0.0021	0.0056	0.0070	0.0185

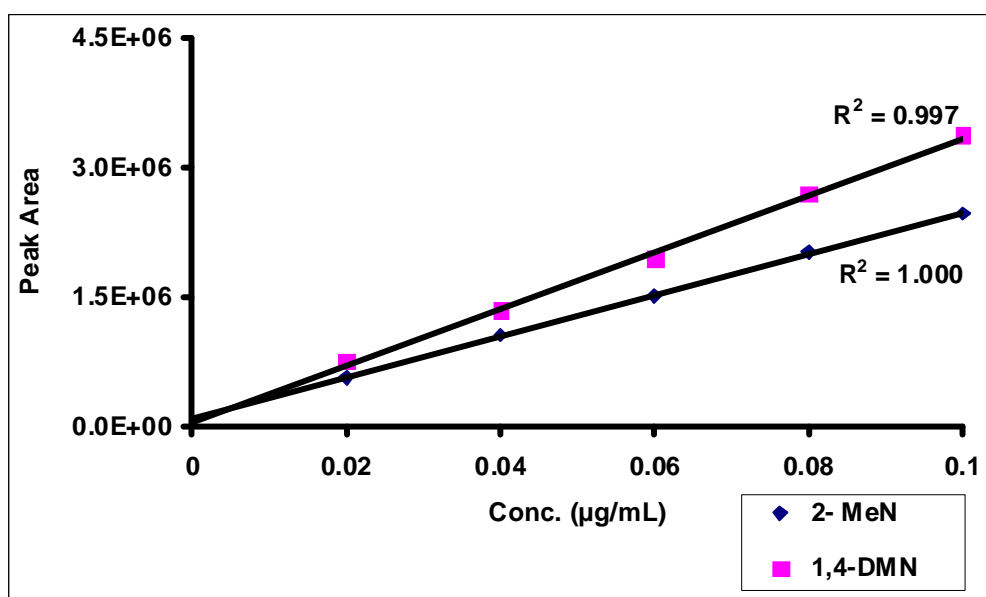


Figure 3:12. Typical calibration graph for 2-MeN and 1,4-DMN at the lower range of concentration 0.02 – 0.1 µg/mL of the mixture.

Experimentally as verification, a mixed standard solution of 0.001 µg/mL of 1,4-DMN and 2-MeN was injected as a test of a low level concentration close to the LOQ. A representative chromatogram in Figure 3:13 shows that both peaks were eluted at this low concentration.

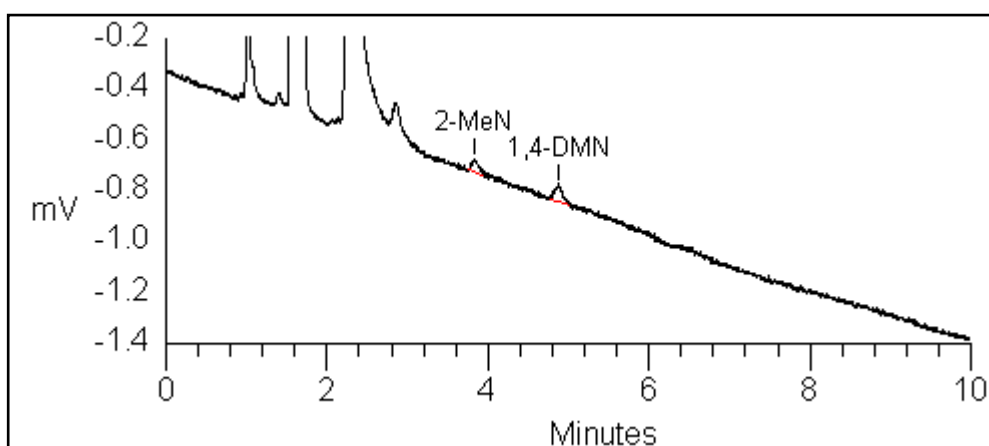


Figure 3:13. Typical chromatogram close to LOQ using 0.001 µg/mL of 1,4-DMN and 2-MeN standard.

3.3.4 Conclusion

During this work, it was essential to overcome the global shortage of acetonitrile solvent and choose an alternative in order to complete this study. For this purpose, methanol was selected as a suitable substitute solvent to develop and validate a routine method of HPLC analysis of 1,4-DMN.

A new isocratic reversed phase HPLC-UV method was presented for the analysis of 1,4-DMN and its internal standard 2-MeN using methanol as a solvent for standards and mobile phase preparations. Good resolution was achieved at methanol concentrations of 50 – 90%, but the shortest run time (6 minutes) was obtained using 90% methanol as the eluent at a flow rate of 1.5 mL/min. Several available columns were tested and the best selection was the Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Spherclone) column.

The HPLC analytical method was successfully validated according to the ICH guidelines (ICH, 1994) in terms of precision, linearity, detection limit and quantification limit. The method achieved high precision through the $RSD\% < 1\%$. Good linearity of the method was confirmed through the coefficient of determination ($R^2 \geq 0.997$). Repeated injections of a 0.01 μ g/mL standard solution produced a lower LOD and LOQ compared to the calibration curve method. The LOD for 1,4-DMN and 2-MeN was found to be 0.0001 and 0.0003 μ g/mL and the LOQ to be 0.0009 and 0.0005 μ g/mL respectively. These values implied that this method is suitable to apply for quantification analysis of these compounds. This method can be applied for the routine analysis of 1,4-DMN residue in potatoes samples and other environmental samples such as water and sediment in quality control laboratories.

3.4 Development and validation of an HPLC method for the analysis of chlorpropham, propham and 3-chloroaniline

3.4.1 Introduction

Chlorpropham (CIPC) is the main sprout inhibitors currently used by potato industry. Propham (IPC) is a herbicide from the same group as chlorpropham; it was applied commercially to prevent sprouting or in combination with chlorpropham, but currently its application is being banned in most countries. Nowadays, for public health and environmental consideration, there is concern about their residues, hence analytical methods are required to analyse the residues of these phenylcarbamates in potato and environmental samples particularly CIPC and its degradation product 3-CA.

HPLC is used to determine carbamate pesticides residues mainly to overcome the thermal lability problems of these pesticides when using gas chromatography (GC). However, a lack of a specific, sensitive detector hinders a suitable level of separation for a number of pesticides. Adequate sensitivity and excellent specificity can be provided by ultraviolet (UV) or electrochemical HPLC detection (Voyksner et al., 1984). Using HPLC-UV seems to be more appropriate as a final step to analyse phenylcarbamate pesticides (Delgado et al., 2001; Orejuela and Silva, 2004; Soriano et al., 2001; Sun and Lee, 2003; LeDoux, 2011).

In reviewing the literature, two simple RP-HPLC methods with external and internal standards were developed for the determination of CIPC in emulsifiable concentrates (Heras and Sanchezrasero, 1982). Samples were diluted with methanol containing internal standard of 4-nitro-diphenyl ether. The chromatographic conditions were set using 60% methanol, at a flow rate of 2 mL/min giving retention times of 4.4 minutes for CIPC and 5.9 minutes for the internal standard. The sample injection volume was 10 μ L and the detection was set at a wavelength of 240 nm. Calibration and quantification were carried out using pure standards of CIPC to achieve good linearity at a concentration range of between 0.01 and 1.5 g/L with a detection limit for CIPC of 0.00039 g/L. The internal standard method reported slight improvement of the confidence limit and the relative standard deviation relative to the external standard method.

HPLC-UV methods have been used to analyse both CIPC and or propham (IPC) in potato products (Koniger and Wallnofer, 1998; Arribas et al., 2007; Wilson et al., 1981; Orejuela and Silva, 2004). These methods have not included the analysis of CIPC in combination with its degradation products in particular 3-CA. However, an isocratic RP-HPLC method has been used to separate and quantify chlorpropham (CIPC) and its metabolites (4-hydroxy CIPC, 3-chloroaniline and 3-chloroacetanilide) in rat hepatocyte using two mobile phases of 90% and 70% methanol (Alary et al., 1986).

Few documented methods have focussed on the determination of CIPC in combination with its degradation products specifically 3-CA in potato samples using different applications. The determination of CIPC residues and its three metabolites namely; 3-CA, 4-hydroxy-CIPC and para-methoxy-CIPC in potatoes samples treated with CIPC have been performed using gas chromatography with a specific nitrogen-phosphorus detector (FAO and WHO, 2001). Capillary GC-MS has been applied to quantify the residues in low levels (ng/g) of CIPC and two of its degradation products; 3-CA and 3,3-dichloroazobenzen (3,3-DCAB) in potato peel samples taken from several market potatoes (Worobey and Sun, 1987). In addition, aniline metabolites of CIPC have been identified in potato samples using capillary chromatography coupled with laser induced fluorescence detection (Orejuela and Silva, 2005).

To date, there is no isocratic method of RP-HPLC coupled with UV-Vis for the determination of parent pesticide CIPC and its degradation product 3-CA.

The predominant analysis methods for CIPC within the University of Glasgow laboratories are based on GC or HPLC analyses. Acetonitrile solvent for standards and mobile phase preparation is used for the HPLC procedure. Due to the global shortage of acetonitrile (in 2008 – 2009), it was considered necessary to validate a method using an alternative solvent to acetonitrile.

The main objective of this work was to develop and validate an analytical HPLC-UV method for the simultaneous analysis of both CIPC and its metabolite 3-CA using methanol as eluent and for standards preparation.

3.4.2 Materials and methods

3.4.2.1 Materials and standards

For the preparation of standard solutions of chlorpropham (CIPC), propham (IPC) and 3-chloroaniline (3-CA) in methanol and preparation of the mobile phase from methanol and water (see Sections 2.1.1 and 2.1.3 respectively).

3.4.2.2 Equipment

The same HPLC system described in Section 2.1.2 was employed to develop a method for analysing CIPC and 3-CA using IPC as an internal standard with exception using column oven and cooling device at this part.

3.4.2.3 Optimising the separation of CIPC, IPC and 3-CA using different strengths of the mobile phase

The effect of the different concentrations of the mobile phase were investigated to optimise the separation of intended compounds and construct a basic background for developing an HPLC separation method with high resolution and rapid analysis of the eluted compounds. In order to achieve this, several concentrations of methanol (70%, 65%, 62%, 60%, 55% and 50%) in the mobile phase were tested to achieve a good resolution of the mixture of components peaks with the minimum run time. All analyses were performed at a detection wavelength of 210 nm, pump flow rate of 1.5 mL/min, an injection volume of 20 μ L and the column at ambient temperature.

3.4.2.4 Selection of detector wavelength

Experiments were conducted examining two UV wavelengths at 210 and 207 nm to select the detector wavelength that gave high sensitivity and selectivity of the present components in the mixture of the standard solution. Five replicate injections of 1 μ g/mL of the mixture of CIPC, IPC and 3-CA were injected at each wavelength 207 and 210 nm and the mean of the peak area was calculated.

3.4.2.5 Determination of the precision of the standard solutions

Five replicate injections of 1 μ g/mL of a mixture CIPC, IPC and 3-CA were injected to measure the precision of the standard solution.

3.4.2.6 Assessment of the linearity of the calibration curve

The linearity of the calibration curve was tested at three ranges of concentrations (0.02 – 0.1, 0.2 – 1.0 and 2 – 10 µg/mL) prepared as three series of standard solutions of a mixture CIPC, IPC and 3-CA in methanol.

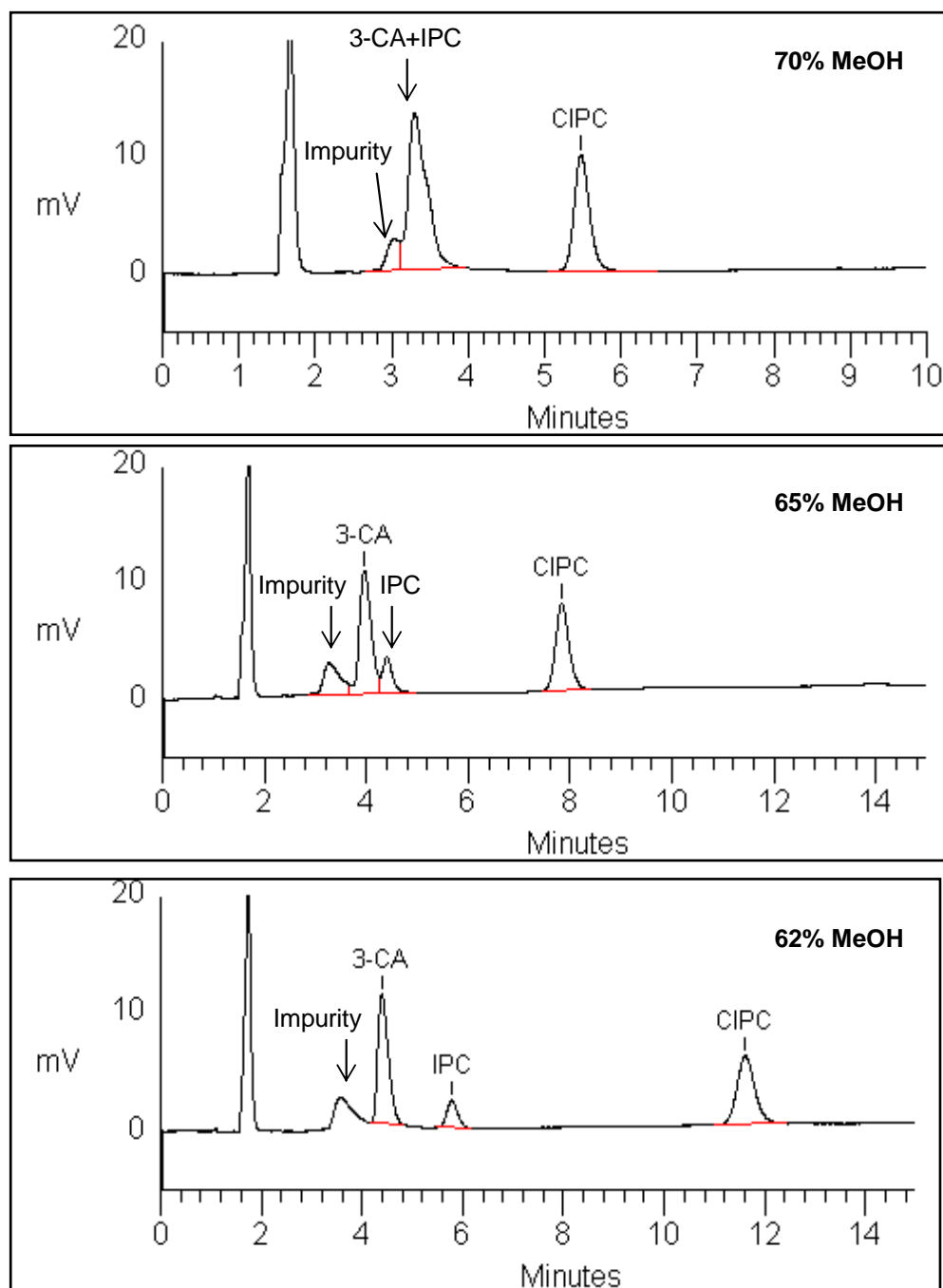
3.4.2.7 Determination of LOD and LOQ

The LOD and LOQ were estimated for three compounds as mentioned in Section 2.1.4.3 applying two approaches including statistical regression of the low concentration range of 0.02 – 0.1 µg/mL and ten replicate injections of mixed solution at the low concentration of 0.05 µg/mL of CIPC, IPC and 3-CA.

3.4.3 Results and discussion

3.4.3.1 Optimising the separation of CIPC, IPC and 3-CA using different strengths of the mobile phase

The chromatographic conditions were set based on an isocratic method using methanol/water as the mobile phase. Protham (IPC) was chosen as the internal standard due to its similarity in structure to chlorprotham (see Figure 4:8) with the only difference being the absence of one chlorine atom in the phenyl ring. The initial chromatographic conditions provided an overview of the identification and optimisation of the separation of CIPC, IPC and 3-CA from the mixture. The HPLC chromatograms in Figure 3:14 illustrate the analysis of 1 µg/mL mixture of CIPC, IPC and 3-CA testing different concentration strengths of the mobile phase.



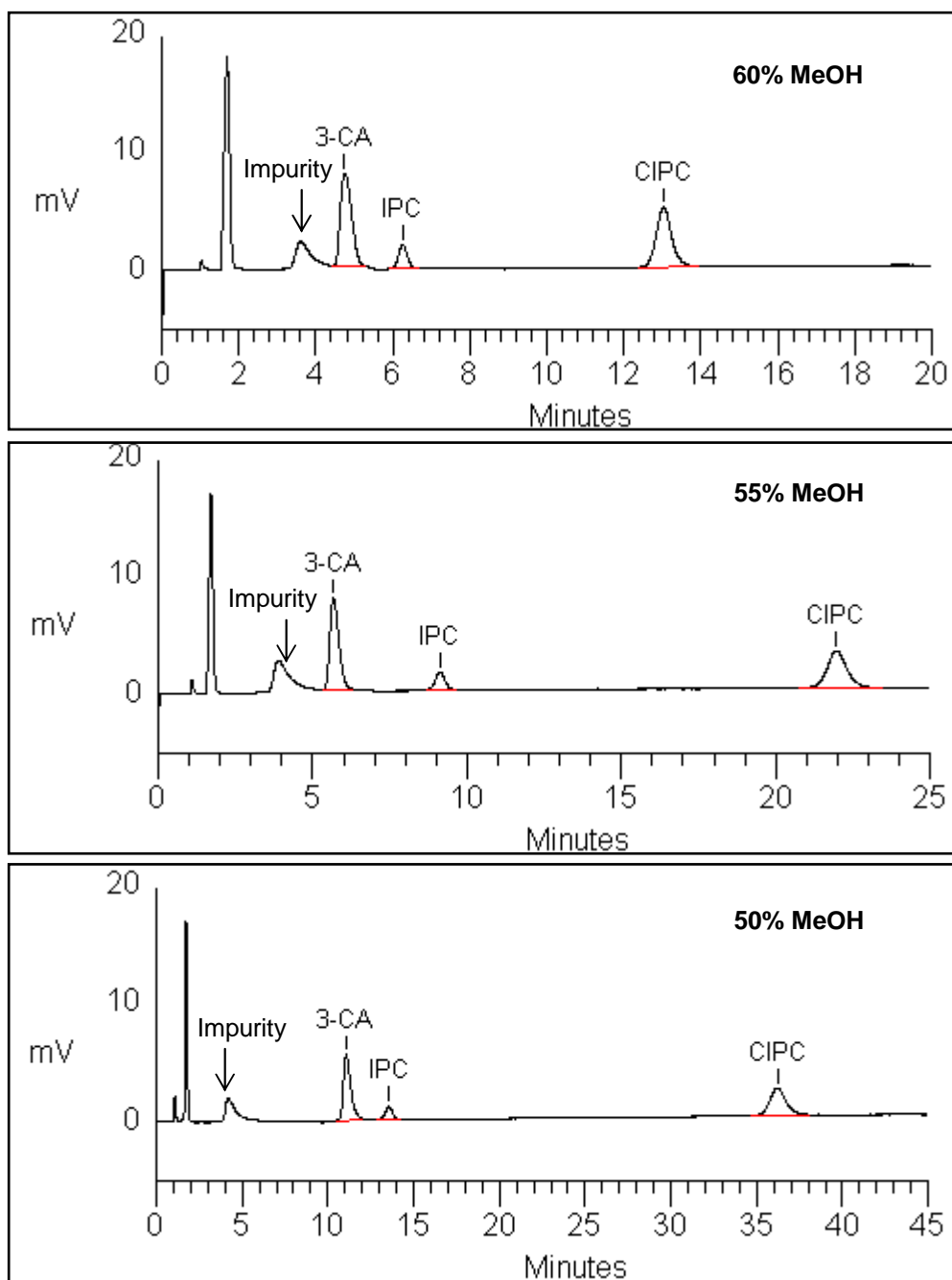


Figure 3:14. Chromatograms of 1 $\mu\text{g/mL}$ mixture of CIPC, IPC and 3-CA at λ_{max} 210 nm using different concentrations of the mobile phase (methanol%) to separate CIPC, IPC and 3-CA at ambient temperature.

This test exhibited good UV absorbance for all compounds at a wavelength of 210 nm, although, the peak height of propham was quite small due to its absorbance being very low at a wavelength of 210 nm compared with its λ_{max} 200 nm (see Section 3.4.3.3). The separation between the compounds was dependent on their polarity. Because of the wide range of polarities between these compounds, the higher polarity compound was eluted

first from the HPLC column. For that reason, the peaks of 3-CA and IPC appeared first and second, respectively, before the final peak of CIPC.

The peaks of eluted compounds were identified in the chromatogram through a comparison of the retention times based on an analysis of a standard mixture and individual reference standards. The same chromatographic conditions were applied during running these standards. In addition, the standards of eluted compounds excluding one compound were analysed to confirm the identity of the peaks and exact retention time of each component.

The chromatogram at 70% methanol showed overlapping between an impurity peak and 3-CA and IPC which co-eluted, whilst CIPC was well resolved. This would suggest that decreasing the mobile phase strength could achieve satisfactory resolution. At 65% methanol/water, a clear improvement was observed when the overlapping peaks began to be resolved from each other and appeared as single peaks.

All three compounds in the mixture were completely resolved at concentrations of methanol less than 65% (62%, 60%, 55% and 50%) but with extremely different run times. At both concentrations of 62% and 60%, the impurity peak (see Section 3.4.3.2) has little effect on the background of the baseline of the 3-CA peak, but this impurity peak can be considered particularly at very low concentration of 3-CA. This lack of the resolution can be solved by selecting a mobile phase strength of 60% but an excessive run time (15 – 20 min) is required. Overall, 62% was considered to provide acceptable resolution of all peaks with a 15 min chromatographic run time.

No effect of the temperature on the separation was noticed. Setting the HPLC column at ambient temperature (~ 20 °C) proved sufficient to obtain optimum separation. In addition, the mobile phase reservoir was insulated against the temperature as noted in Section 3.2.3.2. Standard solutions were kept at a fixed room temperature of 20 °C prior to injection.

3.4.3.2 Impurity peak in the methanol solvent

During the development and validation of HPLC analytical methods, some potential problems should be addressed to mitigate their effects. Impurity peaks are one of the most common problems that arise during the analysis and elute with the intended peaks in the chromatogram (Green, 1996). In sensitive HPLC methods, unexpected peaks are often observed, some can be identified but the source of other peaks can be very hard to trace.

These disturbing peaks can possibly interfere with the analysis and can subsequently influence the quality and reliability of an HPLC method. Thus, noticing these peaks in an HPLC chromatogram requires further investigation to understand their source. Usually, the interference peaks can be traced back to impurities originating from different sources such as sample, mobile phases, buffers, dirty glassware and HPLC systems. Some uncommon artifact peaks have been investigated to understand the sources of their formation. Examples of these peaks were caused by the contamination by the septum of HPLC vial and by the sampling equipment (Yang et al., 2010; Strasser and Varadi, 2000). Occasionally, chemical degradations or unexpected reactions in the sample solutions can result in artifact peaks that are poorly reproducible and hard to predict (Eap et al., 1993; Vogel et al., 2000).

In this work, the source of the impurity peak shown in the chromatograms in Figure 3:14 was studied. This peak was also observed during injection of samples of the mobile phase, water and methanol. The most likely explanation was that this impurity peak might be caused by the methanol itself. To confirm this, samples were tested from several available batches of methanol. These batches were purchased from the same supplier (HPLC grade Fisher Scientific, UK) (see Table 3:10).

Table 3:10. Different batch numbers of methanol with the peak area of the impurity peak.

Methanol batch number	Peak area of the impurity peak
0935126	8545389
0921686	8321945
0919133	8028099
0769625	8284881
0749036	8876873

The chromatogram in Figure 3:15 illustrates that the impurity peak is present in small amounts in all batches. The results of the peak area in Table 3:10 were found to be consistent for all batches. It is also suggested that this impurity may be caused by the presence of dissolved oxygen in methanol (Bandar Al-Sehli, personal communication).

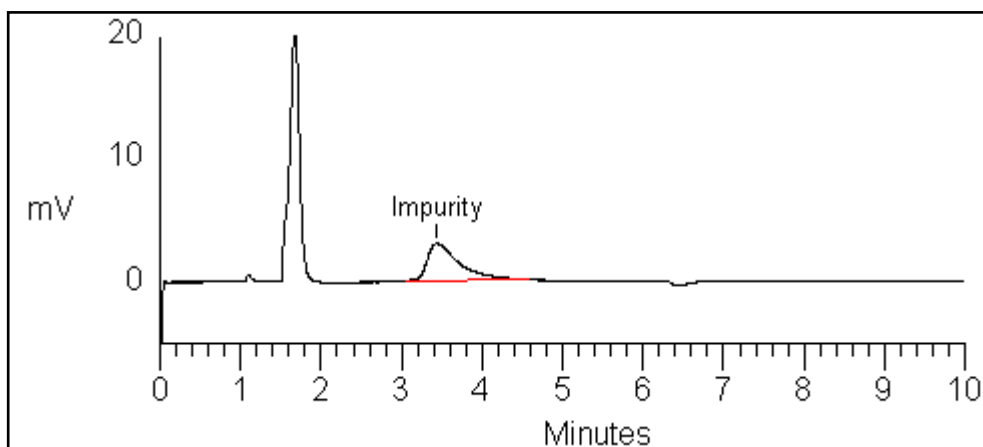


Figure 3:15. Typical chromatogram illustrating the impurity peak present in different batches of methanol.

Commercially, a catalytic reaction of hydrogen and carbon monoxide is typically used to produce up to 80,000 gallons daily of methanol. However, these methods caused undesirable impurities in high grade commercial methanol (Marcus and Glikberg, 1985; Williams, 2004). Indeed, the range of organic solvent quality between different suppliers or product batches is a topical issue. Williams (2004) investigated the susceptibility of gradient LC to the quality of commercially supplied gradient methanol. Contamination in different methanol batches was experienced when testing samples of two separate bottles of the same batch that showed high contamination. Whereas a further five different batches of the methanol exhibited clean blanks applying the same chromatographic conditions to all tests. It was concluded that contamination could occur following the bottling process (Williams, 2004).

An extensive survey to estimate all organic impurities in methanol was carried out using gas chromatography coupled with MS detection (GC/MS) (Guella et al., 2007). Different batches of methanol purchased from different chemical companies were examined. It was found that organic impurities in these batches consisted mainly of dimethyl acetals such as propanone, butanone and pentanone. The polarity of the impurity in the organic solvent plays a significant role in their retention on HPLC columns; highly polar impurities such as amines are strongly retained on alumina columns due to hydrogen bonding with nitrogenous impurities. In contrast, when impurities are less polar they are more difficult to retain (Williams, 2004).

3.4.3.3 Selection of the detector wavelength

Characteristically, in UV-Vis assays all measurements should be assessed at the λ_{max} of the analyte, as that produces a high absorbance response. Choosing a wavelength that is not close to the λ_{max} of the intended compound, can lead to significant changes in the absorbance value producing lower sensitivity measurements and non-linearity of response.

The UV spectra of the studied compounds were measured as described in Section 2.2.3 in order to assess the UV detection wavelength (λ_{max}). Practically, the maximum absorbance of CIPC, IPC and 3-CA could not be characterised accurately in methanol solution thus it is impossible to specify the optimum wavelength λ_{max} . Alternatively, experimental λ_{max} values in acetonitrile solution were estimated. The optimum wavelength λ_{max} found to be 207 nm for both CIPC and 3-CA whereas IPC had a maximum UV absorbance at 200 nm.

The UV spectrum literature addressed considerably different λ_{max} for each of the studied compounds. Hidalgo et al. (1998) measured the UV spectra of CIPC and IPC to give λ_{max} (208, 240) nm (196, 236) nm respectively, whilst, 3-CA absorbs UV light above 290 nm in methanol solution. On the other hand, maximal absorption of CIPC and 3-CA were reported at wavelengths of 277 and 286 nm respectively in aqueous solution (David et al., 1998).

Ideally, the detection wavelength should be at the λ_{max} of the compounds being measured, however, working at a wavelength below or close to the cut off for the methanol eluent (205 nm) can cause increasing baseline noise and a decrease in the linearity response. The SpectraSERIES UV100 detector employed in this method can be operated over the wavelength range 190 – 380 nm with a standard deuterium lamp, giving a wavelength accuracy of ± 1 nm and a bandwidth 6 nm. Hence, a decision was made to set the detector to 210 nm. The initial study of the separation of CIPC and related compounds was carried out using a detector wavelength of 210 nm. This wavelength of 210 nm was also selected according to unpublished work and available HPLC-UV method at the University of Glasgow for the determination of CIPC residues in potatoes samples using acetonitrile as the eluent. In the literature, this wavelength (210 nm) has been set to detect 3-CA using HPLC analysis (Boon et al., 2002).

A comparison was undertaken between the measured λ_{max} of CIPC in acetonitrile (207 nm) and the selected wavelength (210 nm). The aim of this comparison was to assess any significant effect of the wavelength difference on peak area measurement of the intended

compounds in their standard solution. To investigate this, five replicate injections of 1 µg/mL of mixture CIPC, IPC and 3-CA were injected at each wavelength and the mean of the peak area was calculated as shown in the Table 3:11.

Table 3:11. The mean of peak area of each compound of 1 µg/mL mixture of CIPC, IPC and 3-CA at λ_{\max} 207 and λ_{\max} 210.

Compound	Mean Peak Area (n = 5)		
	λ_{\max} 207	λ_{\max} 210	Ratio
CIPC	15716063	14204700	0.90
IPC	5744804	3261071	0.57
3-CA	19683333	14533297	0.74

The experimental results showed a slight decrease in the peak area at 210 nm but this loss of sensitivity is acceptable. Taking all the above considerations, a detector wavelength of 210 nm was selected to avoid the methanol cut off.

3.4.3.4 Summary of chromatographic conditions of the method

In terms of the identification and separation of the three compounds 3-CA, IPC and CIPC from a mixture, the best chromatographic parameters for this method are summarised as follows:

- Column: Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 µm Sphereclone)
- Guard column: Phenomenex[®] Security Guard[™]
- Detector: SpectraSERIES UV100
- Wavelength detection: 210 nm
- Mobile phase: 62 % methanol
- Flow rate: 1.5 mL/min
- Chromatographic run: 15 minutes.
- CIPC retention time: ~ 12 minutes.

- IPC retention time: ~ 6 minutes.
- 3-CA retention time: ~ 5 minutes
- Injection volume: 20 μ L
- Column temperature: ambient ~ 20 °C

3.4.3.5 Determination of precision of standard solutions

The precision in terms of repeatability of five replicate injections was determined for CIPC, IPC and 3-CA through the RSD% to be 0.03, 0.02 and 0.01 respectively. RSD% results indicated acceptable criteria for precision and repeatability was less than 1% (see Section 3.2.3.2).

3.4.3.6 Assessment of the linearity of the calibration curve

Linearity was demonstrated by constructing a calibration curve using five concentration levels of standard solutions for each of the three ranges of concentration. Three calibration curves were plotted for each compound in this test. The coefficient of determination (R^2) was employed to evaluate the linearity of the regression line. The results presented in Table 3:12 show R^2 values were > 0.990 with the exception of IPC at the lowest concentration range (0.02 – 0.1 μ g/mL) where the R^2 was 0.983. This slightly lower value for the coefficient of determination of IPC can be attributed to a low response of this compound at the detection wavelength used (210 nm). From the R^2 values obtained, it can be concluded, that the linearity was acceptable for compounds at the three ranges of concentration tested (see Section 3.2.3.3).

Table 3:12. Coefficients of determination of the calibration curve for studied compounds at the different ranges in concentration.

Compound	Conc. range (µg/mL)	Coefficient of determination (R^2)
CIPC	0.02 - 0.1	0.997
	0.2 - 1.0	0.991
	2.0 - 10	1.000
IPC	0.02 - 0.1	0.983
	0.2 - 1.0	0.995
	2.0 - 10	1.000
3-CA	0.02 - 0.1	0.999
	0.2 - 1.0	0.999
	2.0 - 10	1.000

3.4.3.7 Determination of the LOD and the LOQ

The LOD and LOQ were calculated for the three compounds using two approaches. The results are summarised in Table 3:13 and show no large difference between the calibration curve and the repeated injection approach. The very low LOD and LOQ values for IPC using the repeated injection approach are probably unrealistic in view of the small peak area due to the weak response at 210 nm.

Table 3:13. LOD and LOQ values based on the repeatability injections (n = 10) of 0.05 µg/mL of a mixed standard solution of CIPC, IPC and 3-CA and the statistical data for the calibration curve in the range 0.02 – 0.1 µg/mL.

Assessed approach	LOD (µg/mL)			LOQ (µg/mL)		
	CIPC	IPC	3-CA	CIPC	IPC	3-CA
Calibration curve (0.02 – 0.1 µg/mL)	0.006	0.014	0.003	0.019	0.048	0.010
Injection repeatability (0.05 µg/mL)	0.011	0.001	0.013	0.036	0.002	0.042

Considering the results for the LOD and the LOQ, the method is sufficient to determine CIPC and 3-CA residues in potato and environmental samples. However, the impurity peak discussed in the previous Section 3.4.3.2 overlapped with the 3-CA peak particularly at very low concentrations (~ 0.02 µg/mL). For this reason, it is hard to detect and calculate accurately the peak area of 3-CA at concentrations close to the LOQ using 62% methanol in the mobile phase. Therefore, a 60% concentration of methanol is a better choice at this low level.

3.4.4 Conclusion

No HPLC-UV method for the simultaneous analysis of chlorpropham and its major metabolite 3-chloroaniline in potatoes and environmental samples was documented in the literature. A successful and rapid analytical method was developed and validated for the separation and quantification of these compounds using propham as an internal standard. Furthermore, the project was undertaken during a global shortage of acetonitrile. It was therefore essential to develop a method using an alternative solvent and methanol was selected for this purpose.

The chromatographic conditions required to achieve good separation were 62% methanol at a flow rate of 1.5 mL/min and a detection wavelength of 210 nm with a 15 minute run time. Retention times of approximately 5, 6 and 12 minutes 3-CA, IPC and CIPC peaks respectively were recorded on the Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μ m Spherclone) column at an ambient temperature (~ 20 °C). Although, it would be better to use a 60% mobile phase particularly in the context of a very low concentration of the standards due to the significant interference peak which overlapped with 3-CA peak, in this case, a run time from 15 to 20 minute is required.

Analysis of different batches of methanol confirmed the presence of an impurity in all samples of methanol. This impurity might be caused by contamination during the production and storage of the methanol. Comparison was made between the wavelengths 210 and 207 nm to select the detection wavelength. As 207 nm is close to the cut off for methanol (205 nm), the wavelength of 210 nm was selected.

The method was validated for precision, linearity, the LOD and the LOQ. The precision of replicated injections of a standard solution was measured producing high precision through $RSD \leq 0.03\%$. The method confirmed the linearity of the regression line according to the acceptable criteria of the coefficient of determination (R^2) of the calibration curves (0.990) at three ranges of the concentration for each compound with the exception of IPC at very low concentration. The method recorded lower values of LOD and LOQ and no large difference between the calibration curve and repeated injection approaches. The limit of detection (LOD) values of CIPC and 3-CA were approximately 0.01 μ g/mL whereas the limit of quantification (LOQ) values were approximately 0.04 μ g/mL using repeated injection approach.

The proposed HPLC method is therefore reliable, economical and efficient in terms of the run time. It can be applied to determine and quantify the presence of chlorpropham and its degradation product 3-chloroaniline in potatoes and environmental samples.

At this stage, the proposed method is considered to be suitable for further validation.

Chapter 4: Quantitative analysis and adsorption on laboratory ware of 1,4-DMN, 2-MeN, CIPC and 3-CA in aqueous solutions

4.1 Introduction

The widespread use of pesticides in agriculture and the food industry raises great concern for the environment and human health due to their potential toxicity. Pesticides can be released to the environment via air, soil, water, crops and animals due to their specific characteristics of water solubility and volatility. There is potential for pesticides to adsorb onto soil and be persistence (Tiryaki and Temur, 2010).

Increasing public concern regarding the residues of potato sprout inhibitors mainly 1,4-DMN, chlorpropham and its degradation product 3-CA in wash water during potato processing have compelled researchers in the potato industry to investigate their distribution and fate in the environment. Reliable analytical methods are required for the determination of the residue levels of these pesticides in potato wash water and sediment. However, in some circumstances the accuracy of residue quantification is influenced by many critical factors, which can significantly change the analytical results.

Experimentally, quantitative determination of the intended compounds in environmental samples necessitates using sample containers for sample collection, transportation and storage and different laboratory ware for the extraction procedure. A limiting factor in the quantitative determination of many organic compounds is adsorption onto the wall surfaces of these containers. This phenomenon decreases the concentration of the intended compounds in solution and affects the accuracy of the analytical results (Day and Kaushik, 1987; Sharom and Solomon, 1981).

Generally, the majority of laboratory containers have some ability to adsorb organic compounds and the amount adsorbed is considerably affected by the surface material (Manoli and Samara, 1999). Typically, non-polar hydrophobic compounds in aqueous solution have a strong affinity for adsorption due to their low solubility. Characteristically, the type of material that a container is made from may cause a major impact on the magnitude of adsorption. Several studies have been reported on the loss of pesticides and hydrophobic compounds in aqueous solution due to adsorption. Laboratory studies

conducted by Sharom and Solomon (1981) involved the adsorption of permethrin and other pesticides onto glass and several plastic materials such as polyethylene and polyvinylchloride. A similar study pointed to the loss of dinitroaniline herbicides in aqueous solution and the difficulty in maintaining a constant concentration due to adsorption onto the glass surface when stored in glass containers (Strachan and Hess, 1982). An adsorption study of the lipophilic fenvalerates, as an example of pyrethroid group of insecticides, suggested that transfer of this synthetic compound from water to the sides of glass beakers (100 mL), accounted for losses of between 25 and 33% at different concentrations over a 48 hour period (Day and Kaushik, 1987).

It should be pointed out that the chemical loss from solution may also be attributed to their instability and reaction with container surfaces in addition to adsorption processes. This was indicated by preliminary studies on the commercial fungicide oxycarboxin by Stanton, (1987). This study illustrated that storing solutions in plastic containers can reduce the loss of this compound, whereas storing in soda glass containers showed a reduction in the loss rate of oxycarboxin compared with using containers made from borosilicate glass. This study also suggested that the glass surface played a major role in the degradation rate of the oxycarboxin compound (Stanton, 1987).

Generally, the materials that are recommended for the sampling and storage of environmental samples containing hydrocarbon compounds are borosilicate glass, stainless steel and PTFE (polytetrafluoroethylene) containers. Plastic materials are not preferred as many plastics are porous to volatile compounds causing large losses of these hydrocarbons during transfer and storage. Additionally, biodegradation of some from these compounds can be enhanced by plastic surfaces which facilitate microbial colonisation. Furthermore, some plastic materials such as polyethylene and polypropylene can leach plasticisers e.g. phthalate esters into the sample leading to contamination problems which can later effect the chromatographic analysis of these hydrocarbons (Manoli and Samara, 1999; House, 1994).

Usually sample filtration is required to remove any particles that might lead to interference problems and affect the column efficiency in HPLC analysis. Selecting the appropriate pore size of the filter is important depending on the column packing size to avoid plugging of the column by large size particles (Scheer, 2009). Although many filters possess the same specification their performances are different. Consideration should be taken when choosing a filter regarding the compatibility of the chemical, selecting the right pore size

and the potential adsorption from the chemical solution onto the filter that can produce significant errors in the analysis results. Checking the adsorption is recommended when using any kind of filter for analysis. The potential effect of the filters on both quantitative and qualitative chemical analysis have been reported after observing that many organic compounds can be adsorbed from water solution during filtration (Chiou and Smith, 1970; Mackay and Shiu, 1977). Generally, the extent of the adsorption onto filters depends on the target analyte properties with increasing lipophilicity causing higher adsorption (Gomez-Gutierrez et al., 2007). The solubility of compounds in water also affects the adsorption onto filters, generally less soluble compounds show higher adsorption (Chiou and Smith, 1970).

1,4-Dimethylnaphthalene and the internal standard used in this work 2-methylnaphthalene like other PAHs are hydrophobic organic compounds and their low water solubility can result in high persistence in the environment (Lu et al., 2008). Several processes can influence the accuracy and the precision of the quantitative analysis of these hydrophobic hydrocarbons in aqueous medium including evaporation of volatile components, chemical reaction, biodegradation and incomplete equilibration leading to considerable analytical interference associated with the analysis of these compounds. Furthermore, loss of PAH compounds due to adsorption from static solution to the surfaces of glassware must be considered as well (May et al., 1978; Wolska et al., 2005). Thus, it is crucial to identify the sources of errors and subsequently to find better procedures to remove and reduce their impact prior to analysis. Although CIPC and 3-CA are much more polar and soluble in water than 1,4-DMN and 2-MeN their quantification and adsorption onto containers in aqueous solution should also be investigated.

This work is part of a suggested program to investigate the fate of the studied compounds 1,4-DMN, CIPC and 3-CA in the environment. The objectives are to develop reliable methods for quantitative determination of these compounds in potato wash water and sediments samples. The specific objectives of the work in this part include the investigation of:-

- The solubility behavior of these compounds in water.
- The degradation in aqueous solution.
- The quantitative analysis in water solutions.

- The adsorption potential of these compounds onto laboratory ware that are commonly used in sampling and analysis in order to avoid using those tools that adversely affect the quantitative analysis of these compounds in water.

4.2 1,4-Dimethylnaphthalene and 2-methylnaphthalene

4.2.1 Materials and methods

4.2.1.1 HPLC system

The HPLC system used was the SpectraSERIES UV100 (manual injector) system which includes a manual injector Rheodyne model 7125, Cecil 1100 Series pump, thermo separation products SpectraSERIES UV100 detector and Dionex peaknet software (second HPLC system in Section 3.2.2.2).

4.2.1.2 Chromatographic conditions

Chromatographic conditions were set using a Jones chromatography column (Hypersil ODS 5 μm , 250 mm x 4.6 mm) at ambient temperature, 70% acetonitrile mobile phase at a flow rate of 1.5 mL/min and 10 minute run time. The retention times of 2-MeN and 1,4-DMN were 5.5 and 7 minutes respectively. The injection volume of the sample was 20 μL and the detector wavelength 228 nm.

4.2.1.3 Preparation standard solutions

Standard solutions of 1,4-DMN and 2-MeN in ACN were prepared from a mixed stock solution of 1000 $\mu\text{g/mL}$. The stock solution was also used to prepare working standards solutions in water. To prepare an aqueous standard of 1 $\mu\text{g/mL}$ of 1,4-DMN and 2-MeN, 1 mL from the stock solution was made up to 1000 mL with deionised water and mixed using a magnetic stirrer for 24 hours to reach equilibrium. The 1 $\mu\text{g/mL}$ aqueous standard therefore contains 0.1% ACN.

4.2.1.4 Investigation of the solubility of 1,4-DMN in water

To determine the minimum time required for complete solubility of 1,4-DMN in water solution, three replicates of 1 $\mu\text{g/mL}$ of 1,4-DMN in deionised water (0.1% ACN) were prepared and stirred with a Teflon coated magnetic bar for six hours. 5 mL from each continuously stirred replicate was taken at different times (0, 1, 2, 3, 4, 5 and 6 hours) and

injected twice into the HPLC system. In addition, samples from these solutions were injected after 48 and 96 hours of continuous stirring at 20 °C.

4.2.1.5 Degradation of 1,4-DMN and 2-MeN in aqueous solutions

Degradation of these compounds in aqueous solution was investigated. Two replicates of 1 µg/mL (0.1% ACN) of a mixed solution of 1,4-DMN and 2-MeN were prepared and kept in the incubator at 20 °C . A 5 mL sample was taken every day for analysis over a 10 day period (stirring was performed on the first day only).

4.2.1.6 Comparison of standards prepared in acetonitrile and water

A set of mixed standard solutions of 1,4-DMN and 2-MeN were prepared at different concentrations (1, 5 and 10 µg/µL) in acetonitrile. Another set at the same concentrations were prepared in water (containing 1, 5 and 10% ACN respectively). These solutions were injected immediately in duplicate into the HPLC system to compare between both preparations in acetonitrile and water.

4.2.1.7 Assessment of precision

The precision was measured through ten replicate injections of a mixed standard solution of 0.1 µg/mL of 1,4-DMN and 2-MeN prepared in water (0.1% ACN). The RSD% of the peak area of both compounds was calculated.

4.2.1.8 Calibration curve for standard solutions

Two sets of mixed standards of 1,4-DMN and 2-MeN were prepared in water at concentration ranges of 0.02 – 0.1 and 0.2 – 1.0 µg/mL to assess the linearity of the calibration curve (see Section 2.1.4.2). Each standard was injected in duplicate.

4.2.1.9 Determination of the LOD and the LOQ of 1,4-DMN and 2-MeN in aqueous solutions

The LOD and LOQ of 1,4-DMN and 2-MeN were assessed in water solution by two approaches as explained in Section 2.1.4.3.

4.2.1.10 Adsorption of 1,4-DMN and 2-MeN onto the laboratory ware in aqueous solutions

1. Adsorption onto glass and plastic containers

Different laboratory containers and transfer tools including glass, bottles, flasks, plastic, filters and syringes were tested to investigate the potential of the adsorption of 1,4-DMN and 2-MeN from their aqueous solution onto surfaces of the laboratory ware. The test involved filling glass and plastic containers (each one in triplicate) with an aqueous solution (0.1% ACN) of 1 µg/mL of 1,4-DMN and 2-MeN. The replicates were left standing (~ 15 minutes) prior to analysis. The adsorption potency was expressed as a percentage recovery representing the 1,4-DMN and 2-MeN remaining in the solution.

2. Adsorption onto filters

Different filter papers, membranes and syringe filters were examined. All filters with the exception of the syringe filter were soaked in 10 mL of the aqueous standard solution of 1,4-DMN and 2-MeN placed in screw cap vials (20 mL). 10 mL of the standard solution was left in the vial as a control without filters. The procedure for examining the syringe filter (supor membrane 32 mm, 0.2 µm) included using a plastic 20 mL syringe. 10 mL was withdrawn from the standard solution into the syringe. The solution in the syringe was analysed first as a control then the syringe filter was connected to the syringe and the solution was pushed into a screw cap vial for analysis by HPLC. Each test included three replicates and the test solution and the control were injected in duplicate.

3. The effect of contact time on adsorption

The effect of contact time on adsorption was also investigated by taking a sample after different time intervals. Five screw top jars (100 mL) were washed with decon, 1 M NaOH and ACN. After drying, 10 mL of the mixed standard solution of 1 µg/mL of 1,4-DMN and 2-MeN (0.1% ACN) prepared in deionised water was added. The jars were thoroughly sealed and then samples were taken from each jar at different times. Each sample was injected in duplicate and the mean of the peak area was calculated. The standard in the volumetric flask was injected between the jar samples and the mean of the peak area of five injections was calculated.

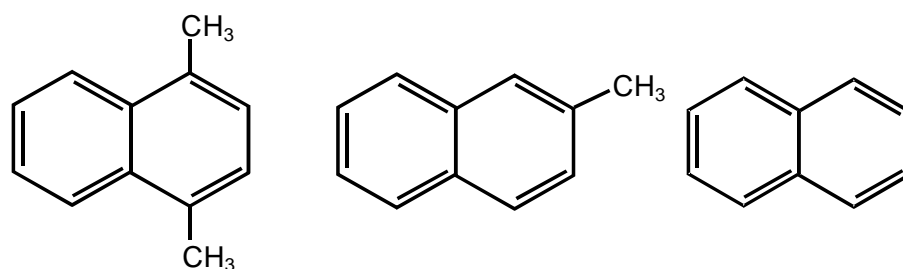
4. Treatment of glass containers to reduce adsorption

To minimise the potential of chemical adsorption on glassware, volumetric flasks (50 mL) were treated by rinsing with different solutions using Decon 90, 1 M NaOH, 1 M H₂SO₄ and ACN.

4.2.2 Results and discussion

4.2.2.1 Investigation of the solubility of 1,4-DMN in water

Principally, the solubility of organic compounds in water depends on their interaction with water molecules by mechanisms like hydrogen bonding or dipole-dipole interactions. PAHs have a low rate of dissolution in water and low aqueous solubility. Generally, the decreasing solubility of PAHs and the increasing hydrophobic properties are associated with increasing number of benzene rings and molecular length (Juhasz and Naidu, 2000). However, several compounds have the same carbon number and molecular length but their solubilities are different (May et al., 1978). 1,4-DMN and its internal standard 2-MeN like all polyaromatic hydrocarbons are slightly soluble in water tending to be present as droplets in suspension when added to water (see Figure 4:1).



1,4-Dimethylnaphthalene

2-Methylnaphthalene

Naphthalene

Physical form	Liquid	Crystal	Crystal
Molecular formula	C ₁₂ H ₁₂	C ₁₁ H ₁₀	C ₁₀ H ₈
Molecular weight (g/mole)	156.23	142.20	128.18
Water solubility (mg/L) at 25°C	11.4	24.6	31
Vapor pressure (mm Hg) at 25°C	0.0214	0.055	0.085

Figure 4:1. Physiochemical data of the polycyclic aromatic hydrocarbons compounds 1,4-dimethylnaphthalene, 2-methylnaphthalene and naphthalene (SRC, 2011).

As a compound in this group, the aqueous solubility of 1,4-DMN has been measured by Mackay and Shiu (1977) to be 11.4 ± 0.1 mg/L at 25 °C. A saturated solution was prepared by adding an excess weight of the compound to distilled water which was stirred for 24 hours using a Teflon coated magnetic bar. It was then left to settle for 48 hours before measurement (Mackay and Shiu, 1977).

To prepare a standard solution of 1,4-DMN in water, its low solubility does not allow mixing instantaneously with water making it difficult to dissolve and reach equilibrium. Additionally, the time required for complete dissolution according to the work done by Mackay and Shiu (1977) is a time consuming process for preparing many standard solutions for the purpose of this investigation. Thus, the suggestion was to prepare a stock solution first in acetonitrile. Then this stock solution in acetonitrile was used to prepare the required concentrations of aqueous standards by adding an appropriate volume to deionised water in a volumetric flask. Although, there would be a small concentration of

acetonitrile present, there is concern about how easily the 1,4-DMN in the two solutions (ACN and water) would mix.

To assess the minimum time required to obtain good dissolution of 1,4-DMN standards from ACN into water, measurements were made of replicate standard solutions at different times stirring. The response of peak area was plotted against time as shown in Figure 4:2.

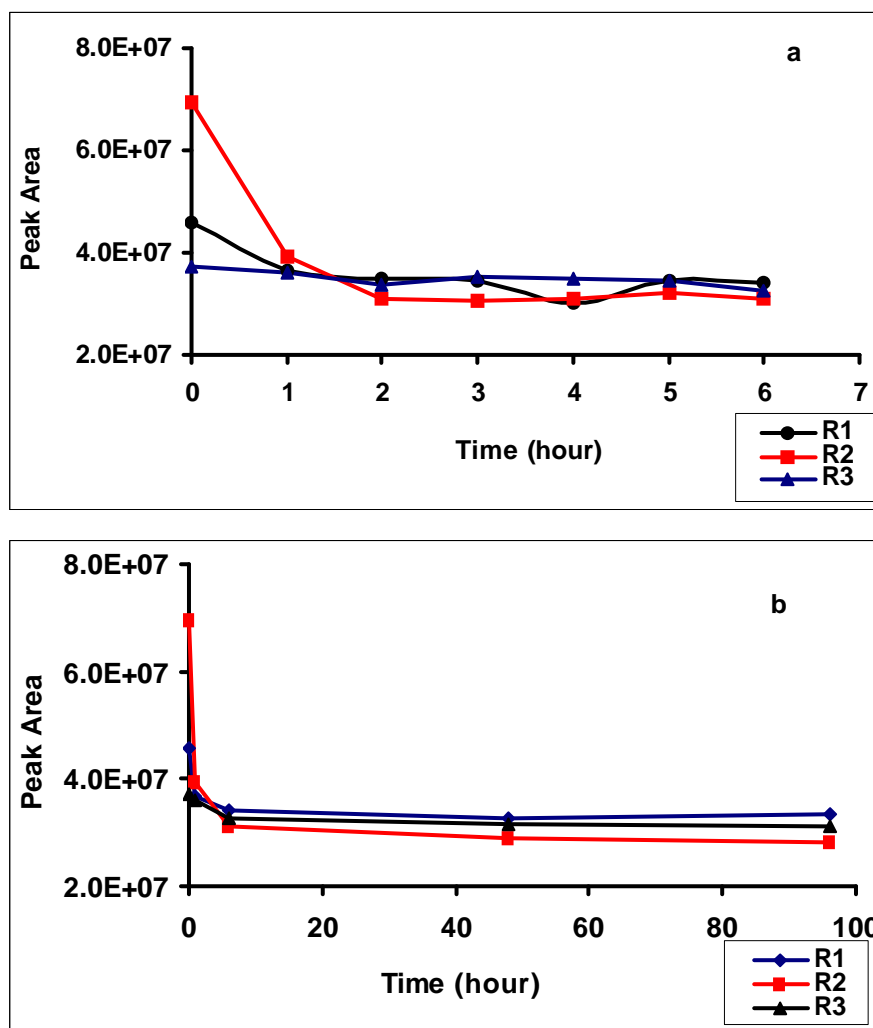


Figure 4:2. The mean of the peak area of three replicates of 1 µg/mL 1,4-DMN in deionised water (0.1% ACN) in different mixing time: a- during the day and b- different days.

This figure illustrates that 2 – 3 hours mixing with continuous stirring by magnetic stirrer at 25 °C temperature was sufficient to prepare a 1 µg/ mL standard solution of 1,4-DMN in aqueous solution. After this time, no change in the peak area was observed even after 4 days mixing. Although stirring should have no effect on the 1,4-DMN solubility it can increase the interaction of the 1,4-DMN with water consequently increasing the speed of reaching equilibrium.

4.2.2.2 Degradation of 1,4-DMN and 2-MeN in aqueous solutions

The degradation of many polycyclic aromatic hydrocarbons in aquatic environments can take place and decrease their concentrations as result of biological degradation or photochemical oxidation (Swietlik et al., 2002). Some PAHs are subject to degradation by microorganisms: bacteria, fungi and algae, in particular those lower molecular weight compounds which contain three or less fused benzene rings (Juhasz and Naidu, 2000; Seo et al., 2009). Practically, the loss of some PAHs in water and darkness was noticed after 21 days, this loss varied from 22% to 41% depending on the type of compound, solution composition and the exposure conditions (Swietlik et al., 2002).

The instability of PAH standards at low concentration could be of concern. In this work, an investigation was conducted to study the possibility of the degradation of 1,4-DMN and 2-MeN in deionised water and during storage of standard solutions in the dark. Two replicates were analysed daily for ten days. The mean peak area of each compound was calculated and plotted against time as shown in Figure 4:3.

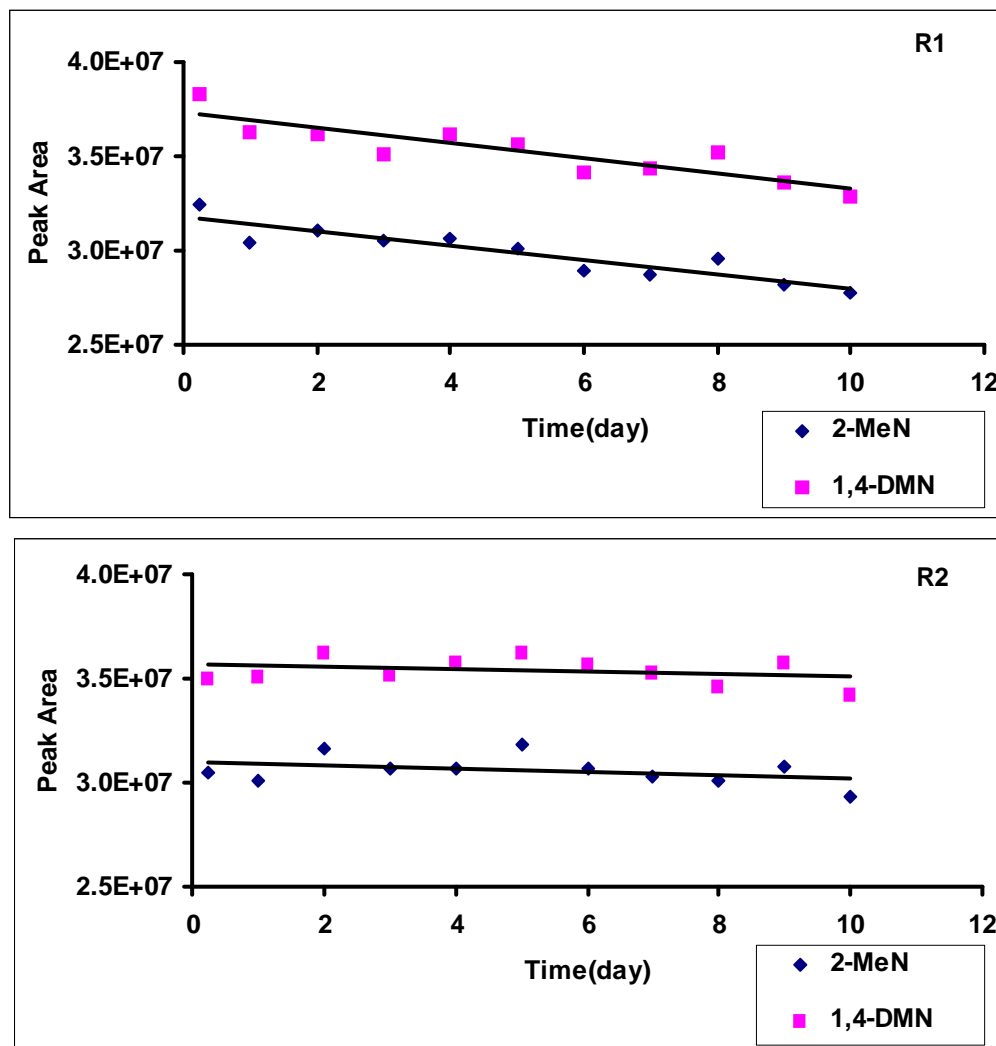


Figure 4.3. The mean of the peak area of two replicates (R1 and R2) of 1 µg/mL of mixed solution of 1,4-DMN and 2-MeN in deionised water (0.1% ACN) on different days.

Treating the data to a simple linear regression using Minitab, the results showed non significant degradation ($p > 0.05$) of 2-MeN and 1,4-DMN for replicate R2 whereas replicate R1 indicated a significant degradation ($p < 0.001$) of both compounds. It should be noted that these were not sterile solutions so it is possible that replicate one was affected by biological decomposition while the other was not.

In conclusion, these working solutions should be kept in the fridge at 4 °C temperature and used for short time only to avoid this kind of degradation.

4.2.2.3 Comparing standards prepared in acetonitrile and water

To compare standard solutions prepared in organic solvent (ACN) and water, this study was carried out by preparing different concentrations (1, 5 and 10 µg/µL) of mixed solution of 1,4-DMN and 2-MeN in acetonitrile and in water containing 1, 5 and 10 % ACN respectively. Analysis of these solutions was performed by HPLC and

chromatograms showed peaks with good shape and stable retention time as shown in Figure 4:4.

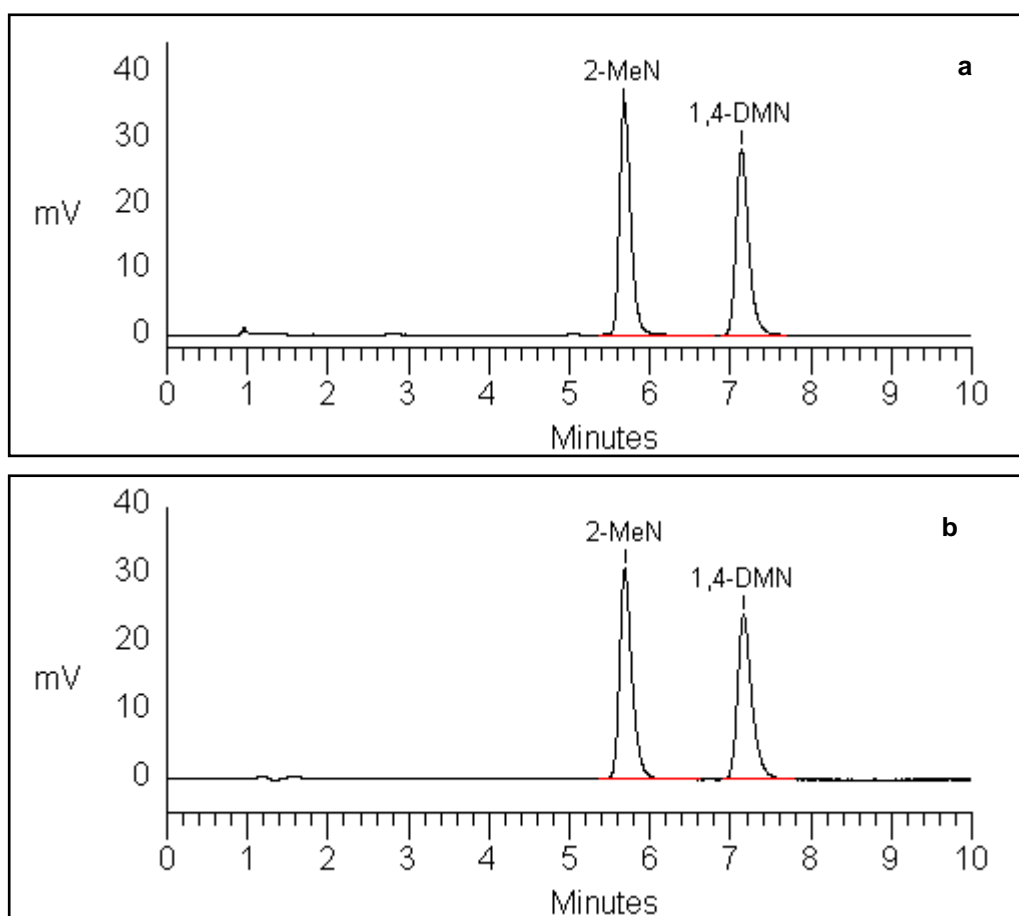


Figure 4:4. Chromatograms of 1 µg/mL mixture of 1,4-DMN and 2-MeN prepared in: a- water (0.1% ACN) and b- 100% ACN.

Additionally, the results of a two sample t-test of the peak area for 2-MeN (see Table 4:1) showed no significant difference ($p > 0.05$) at all three concentrations of (1, 5 and 10 µg/µL) between the preparation in water and ACN. However, for 1,4-DMN preparation there was a significant difference ($p < 0.05$) at all three concentrations. Although, statistically significant, practically this was a small random variability that could be due to volumetric error in the preparation. Therefore, these results confirm the reliability of using these solutions for subsequent experiments.

Table 4:1. The mean of peak area and the t-test for each compound in the mixture of 2-MeN and 1,4-DMN prepared in acetonitrile and aqueous solutions at different concentrations.

Conc. ($\mu\text{g/mL}$)	Mean peak area of 2-MeN (n = 2)		t-test	Mean peak area of 1,4-DMN (n = 2)		t-test
	Acetonitrile	water		Acetonitrile	water	
1	33189694	34433598	NS*	29982648	31216848	S*
5	154184703	155826052	NS	146041669	141575039	S
10	291259545	294843740	NS	292822597	302510876	S

NS*: no significant difference ($p > 0.05$), S*: significant difference ($p < 0.05$)

It should be pointed that the aqueous standards in this work contained a high percentage of acetonitrile (1, 5 and 10% ACN), however, it is possible to prepare standards with lower concentrations of acetonitrile (0.01, 0.05 and 0.1%) using different dilution methods.

4.2.2.4 Assessment of precision

The RSD% values of 1,4-DMN and 2-MeN were 1.8 and 2.1 respectively for ten replicate injections of a mixed solution of 0.1 $\mu\text{g/mL}$ in water (0.1% ACN). The RSD% values were higher than 1 which was selected as the precision criteria in this study. The low concentration provided poor signal to noise. However, as mentioned in Section 3.2.3.2, an RSD% of $\leq 5\%$ is acceptable for a method at low level concentrations close to the limit of detection.

4.2.2.5 Calibration curve for standard solutions

The linearity of standard preparations was tested. The calibration curves were constructed by plotting the peak area of each compound against the corresponding concentrations. The five points of the regression line (each point in duplicate) offered good linear behaviour in the ranges 0.02 – 0.1 and 0.2 – 1.0 $\mu\text{g/mL}$. The coefficient of determination (R^2) values as shown in the figures below were found to be acceptable and close to 0.990, which was the R^2 selected for the linearity criteria in this study (see Section 3.2.3.3).

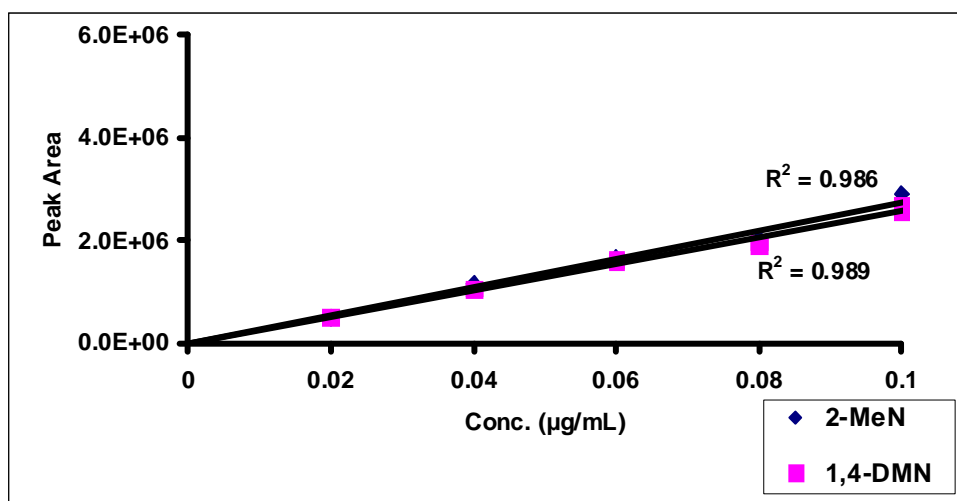


Figure 4:5. Calibration graph for 2-MeN and 1,4-DMN at range 0.02 – 0.1 µg/mL in aqueous solution.

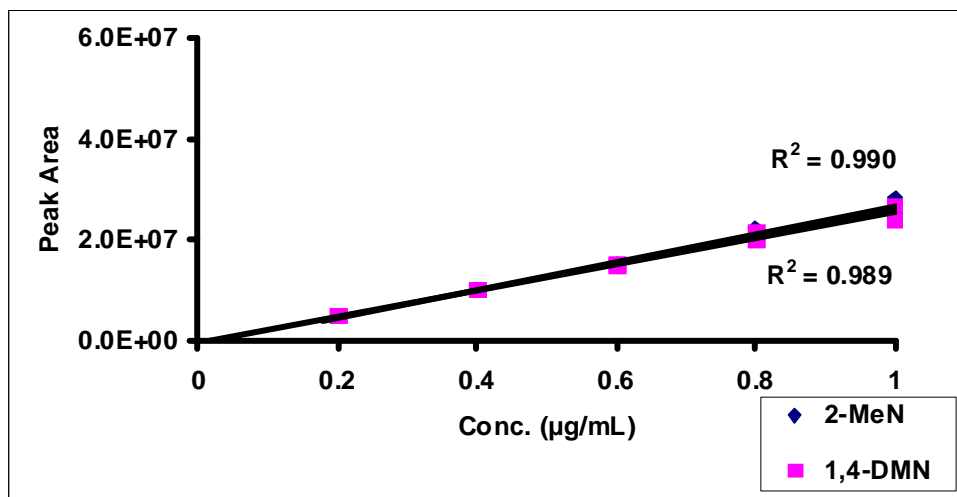


Figure 4:6. Calibration graph for 2-MeN and 1,4-DMN at range 0.2 – 1.0 µg/mL in aqueous solution.

4.2.2.6 Determination of the LOD and LOQ of 1,4-DMN and 2-MeN in aqueous solutions

Two approaches to measuring the LOD and LOQ were applied based on the standard deviations of ten replicate injections of a low concentration of 0.1 µg/mL and the standard deviation of the calibration curve at the low range 0.02 – 0.1 µg/mL.

Table 4:2. LOD and LOQ values based on the statistical data for the calibration curve in the range 0.02 – 0.1 µg/mL and repeated injection (n = 10) of 0.1 µg/mL of mixed 2-MeN and 1,4-DMN in aqueous solution.

Assessed approach	LOD (µg/mL)		LOQ (µg/mL)	
	2-MeN	1,4-DMN	2-MeN	1,4-DMN
Calibration curve (0.02 – 0.1 µg/mL)	0.012	0.010	0.039	0.033
Injection repeatability (0.1 µg/mL)	0.006	0.005	0.021	0.018

As seen in the table, the results of using the two different approaches indicate that the repeated injections approach provided slightly lower and more realistic values than the calibration curve approach. The LOD and LOQ values were close to those for standards prepared in ACN as described in Section 3.2.3.4. This confirmed that there was no difference between the standards prepared in water or ACN.

4.2.2.7 Adsorption of 1,4-DMN and 2-MeN onto the laboratory ware in aqueous solutions

The results below report the loss of compounds expressed as a percent recovery which represents the concentration of 1,4-DMN and 2-MeN present in the solution after contact with the laboratory ware compared with the original solution. The standard solution was kept in a volumetric flask and sealed to prevent any volatilisation. As concluded in Section 4.2.2.3, no big difference was found between the preparation of the investigated compounds in ACN and water, thus confirming that there was no adsorption of the compounds in water onto the surface of the volumetric flask. Additionally, injecting the standard solution at intervals over a four hours period showed good precision of peak area ($RSD\% < 1$) for five replicate injections confirming that there was no loss during the experiment.

1. Adsorption onto glass and plastic containers

The adsorption results for different glass containers are shown in Table 4:3.

Table 4:3. The recovery% and RSD% values of 2-MeN and 1,4-DMN from different kinds of glass containers.

Glass containers	Material	2-MeN		1,4-DMN	
		Recovery %	RSD% (n = 3)	Recovery %	RSD% (n = 3)
Volumetric flask (50 mL)	Borosilicate	95	0.3	98	0.7
Reagent bottle (100 mL)	Borosilicate	89	1.7	91	2.6
Quick fit conical flask (100 mL)	Borosilicate	98	0.6	98	0.7
Quick fit test tube (10 mL)	Borosilicate	98	3.1	100	3.7
Auto sampler vial (2 mL)	Soda	96	2.3	98	1.5
Screw vials (20 mL)	Soda	97	0.8	98	1.0
Screw top jar (100 mL)	Soda	66	10.3	68	10.4

The results show the percent recovery from each container is more than 89% for both types of glass containers giving acceptable precision with the exception of the soda glass screw top jar which exhibited a much lower recovery and high RSD%.

No relation was observed between the types of glass container materials (borosilicate and soda) and the adsorption potency. However, it was shown by Thakker et al. (1979) that the adsorption of hydrophobic compounds onto glass surfaces is significantly stronger onto Pyrex glass (Pyrex is the trade name of borosilicate). The authors speculated that this was due to the heterogeneity of the surfaces of this kind of glass.

The good recovery in the present study might be due to the presence of the small concentration of acetonitrile (0.1%) in the aqueous solutions which might reduce the potential to adsorb onto the surface of the glass. In conclusion, some containers were identified (recovery $\geq 95\%$) which could be used in the quantitative measurements of 1,4-DMN and 2-MeN in water samples.

In contrast, the plastic materials caused a much larger decrease in the recovery of 1,4-DMN and 2-MeN and with high variability as seen in the Table 4:4.

Table 4:4. The recovery% and RSD% values of 2-MeN and 1,4-DMN from different kinds of plastic materials.

Plastic containers	2-MeN		1,4-DMN	
	Recovery%	RSD%	Recovery%	RSD%
	(n = 3)		(n = 3)	
Bottle Polystyrene	92	1.5	93	0.6
Bottle Polypropylene	62	3.1	62	3.2
Bottle HDPE	44	10.1	45	10.7
Plastic syringe (BD Plastipak)	59	16	56	19
Micro pipettor tip	34	5.3	32	5.7

Polystyrene gave the best recovery of both compounds (92 – 93%) with good precision. The low recovery results may be due to the non-polarity of these polyaromatic hydrocarbons, which are insoluble in water because they are not able to break the forces between water molecules. Adsorption is perhaps favoured because the non-polar, sparingly soluble hydrocarbons in plastic containers have a tendency to partition onto the plastic container walls. The adsorption mechanism may be irreversible and most often multilayer, these several layers are part of a highly organised system in the adsorbed phase (Thakker et al., 1979).

Strachan and Hess (1982) speculated that hydrocarbon compounds bind to plastic materials by physical phenomena not related to chemical reactions. A combination of electronic and electrostatic interactions of the aromatic ring with hydrophobic properties of plastic may result in the adsorption of these compounds from their aqueous solutions preferentially onto these plastic materials.

2. Adsorption onto filters

The systematic investigation in this work was to assess the possible adsorption of 1,4-DMN and 2-MeN from water solution onto several types of widely used filters (filter papers and membrane filters). No work was found in the literature concerning these materials and studied compounds. The results are shown in Table 4:5, the percent recovery of each compound was observed to vary with the type of filter. The supor membrane filter and

syringe filter offered low recovery with high RSD%. However, there was a smaller loss of both compounds with Whatman filter paper and glass microfiber filters.

Table 4:5. The recovery% and RSD% values of 2-MeN and 1,4-DMN in their solution after adsorption on different filters.

Filter specification	2-MeN		1,4-DMN	
	Recovery	RSD%	Recovery	RSD%
	%	(n = 3)	%	(n = 3)
Supor membrane filter (47 mm, 0.2 μ m)	32	69.2	34	65.4
Whatman filter paper (grade 1, 2 cm)	81	7.1	80	6.2
Glass microfiber filter (GF/C, 25 mm)	77	11.8	80	10.0
Syringe filter (supor membrane 32 mm, 0.2 μ m)	38	57.4	27	71.4

According to these results, filtration may not be possible for the analysis of 1,4-DMN and 2-MeN because they can be lost in this step owing to adsorption onto the filters. Therefore, additional filters such as PTFE, nylon, cellulose nitrate, mixed cellulose esters and polycarbonate should be tested. Using a very large volume of sample solution to saturate the adsorbed sites of filter might be a possible alternative. Centrifuging could also be investigated using glass centrifuge tubes but that must be compatible with HPLC column packing size (less than 0.2 μ m) to avoid plugging of column by large size particles. Typically, for centrifuging large particle sizes, several factors are required such as a high centrifuge speed, high-density difference between the solid materials and liquid in solution and low viscosity.

As one from this group of polyaromatic hydrocarbon, naphthalene may represent a good example (see Figure 4:1). This compound in water solution was found to be adsorbed by filter disks (17.5 mm diameter) at two pore sizes 0.22 μ m and 0.025 μ m; the percentages adsorbed from 3 mL were 86 and 98% respectively from the initial concentration of naphthalene solution. The high adsorption demonstrated the role of van der Waals force as a mechanism in this adsorption. Additionally, the percent adsorption from the fourth filtration through the same filter of 0.22 μ m was 85% which means the adsorption capacity of filter was still high enough to adsorb the naphthalene after multi filtration of the solution (Chiou and Smith, 1970).

3. The effect of contact time on adsorption

In this study, it was observed that several containers (e.g. screw top jar) had high adsorption for the studied compounds. Thus, it was worth investigating if the length of contact time provides an opportunity for greater adsorption.

The effect of the length of contact time on the adsorption of 1,4-DMN and 2-MeN on soda glass jars was investigated. The results of percent mean recovery versus the contact time with the jar surface are shown in Figure 4:7. These exhibit a decrease in the percent recovery of both 1,4-DMN and 2-MeN with longer duration of the contact time.

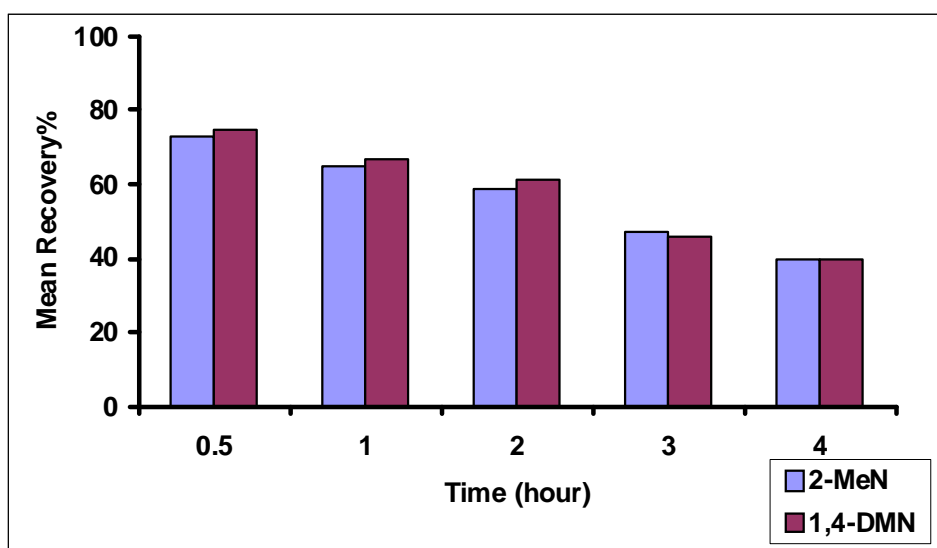


Figure 4:7. The mean recovery% (n = 5) of 2-MeN and 1,4-DMN from using screw top jar (100 mL) at various time intervals.

As can be seen, the recovery of both compounds decreased from 75 to 40 % after 4 hours confirming the role of contact time on adsorption.

To demonstrate a maximum adsorption of 1,4-DMN and 2-MeN onto the surface of the jar for obtaining equilibrium, a series of experiments are also required over longer contact times for the solution in the jar. Furthermore, the effect of many factors on the adsorption should be taken into consideration including shaking, agitation period, temperature and pH of the solution.

Although it was reported by Wolska and co-workers (2005) that the adsorption process of PAHs on the surfaces of glass vessels can take place immediately and that no noticeable changes are observed over time, the study by Sharom and Solomon (1981) showed that the adsorption loss of permethrin during storage onto the surface of glass sample containers increased quickly in the first 24 hours of contact time and then remained constant from 48

to 120 hours. The authors indicated that increasing the sample volume to surface area of contact ratio resulted in decreased adsorption by glass. Low recovery of synthetic pyrethroid insecticides in water samples was also determined during storage in glass containers and extraction using solid-phase membranes (Lee et al., 2002). The loss of all pyrethroid compounds rapidly increased due to adsorption onto glass surfaces, until the concentration became constant at 58 – 72% of the initial concentration. In a series of experiments on the adsorption of trifluralin, which is considered as the model for dinitroaniline herbicides, the amount adsorbed onto the glass surfaces was affected by many factors: the time of the contact, shaking and agitation period (Strachan and Hess, 1982). When the vial contents were left undisturbed, the results reported decreasing of trifluralin in 1% ethanol in water from initial concentration 5 to 0.63 μM after 2 hours. While using continuously shaken vial contents during 2 hours incubation the concentration dropped from 5 to 0.39 μM .

4. Treatment of glass containers to reduce adsorption

Trace impurities on the glassware rather than the container material itself may have some role in adsorption. Thus, the glassware used should be thoroughly cleaned to eliminate or minimise any interference problems and loss of analyte. Several methods are available for cleaning the glassware. For analysis of PAHs in water samples, careful planning is required. Usually, the washing procedure includes using detergent and water as an initial step followed by rinsing with organic solvents such as acetone and hexane to remove any polar and non polar species from the glassware surfaces (Manoli and Samara, 1999). In some cases, heating to 400 °C for 1 hour is recommended for the non-volumetric borosilicate glassware even though thermally stable compounds such as polychlorinated biphenyls (PCBs) may not be eliminated unless rinsing the glassware is subsequently is done with acetone (House, 1994).

In this study, in order to minimise the extent of the adsorption of 1,4-DMN and 2-MeN onto the glassware, various washing procedures were tested using different combinations of Decon 90, 1 M NaOH, 1 M H_2SO_4 and ACN. These treatment procedures were tested on volumetric flasks (50 mL).

Decon 90 is a surface-active detergent used for cleaning and or decontaminating laboratory glassware after dilution with water to 2 – 5%. The glassware is immersed and soaked for 2 – 24 hours based on the contamination problem. For washing laboratory glassware which

is to be used solely for organic compounds if the detergent alone is not sufficient for cleaning then acidic or basic solutions should be used by soaking overnight.

The aim of rinsing the glassware with ACN is to remove non-polar materials. In addition, it is the solvent used in this study for the mobile phase and the standards preparation.

In this work, when the volumetric flasks were washed with Decon 90 alone, or Decon 90 followed by ACN approximately 2 – 6% of the compounds was lost as shown in Table 4:6. Washing these volumetric flasks with Decon 90, then 1 M NaOH or 1 M H₂SO₄ then ACN increased the recovery to above 98% with good precision. New 50 mL volumetric flasks that had never been used before also gave good recovery with good precision whether treated or not. This suggests that it is either contamination or degradation of the glass surfaces that might cause adsorption in the older flasks.

Table 4:6. The recovery% of 2-MeN and 1,4-DMN using different treatments of volumetric flasks (50 mL).

Treatment of Volumetric flask (50 mL)	2-MeN		1,4-DMN	
	Recovery	RSD%	Recovery	RSD%
	%	(n = 3)	%	(n = 3)
Decon 90	95	0.3	98	0.7
Decon 90 + ACN	94	1.4	97	1.4
Decon 90 +1 M NaOH + ACN	101	1.0	102	1.1
Decon 90 +1 M H ₂ SO ₄ + ACN	100	1.6	101	1.8
New flasks without treatment	103	0.1	103	0.1
New flasks + 1 M NaOH +ACN	98	2.0	99	1.8
New flasks + 1 M H ₂ SO ₄ + ACN	98	2.0	99	1.9

These series of experiments show that treatment with sodium hydroxide or sulphuric acid followed by ACN resulted in acceptable recovery of 1,4-DMN and 2-MeN from these volumetric flasks which therefore are a good choice to use for quantitative analysis of these compounds in water samples.

Laboratory studies have reported the effect of using acid and base materials on adsorption by glassware. A study by Farrer and Hollenberg (1953) of successive alkaline treatment of volumetric flasks with sodium hydroxide showed no adsorption of thiamine. In addition, no adsorption of thiamine was shown onto glass under conditions of pH 1 using

concentrated hydrochloric acid in the solution. Therefore, the authors were using an acidified solution in their laboratory and treating the glassware with alkaline solution every three months (Farrer and Hollenberg, 1953).

The pH of the solution has a pronounced effect on the magnitude of adsorption on the surfaces of the containers. This was illustrated through studying the adsorption of methotrexate drug in alcoholic solutions onto glassware and syringes (Chen and Chiou, 1982). It appeared that the adsorption was reduced at lower (pH 2 – 4) or higher (pH 8 – 9) values.

A study of the adsorption of the hydrophobic amine drug (α -[(dibutylamino)methyl]-6,8-dichloro-2-(3',4'-dichlorophenyl)-4-quinolinemethanol monochloride) onto surfaces of different types of containers (Thakker et al., 1979) showed that preparing solutions of water-methanol (1:1) in 0.01 M H₂SO₄ from this drug minimised the adsorption onto the surfaces of the containers. The observation was that using polyfluoroethylene beakers over 10 hours in the presence of 0.01 M H₂SO₄ resulted in the loss of only 20% of the drug but replacing the acid with buffer solution of phosphate at pH 5.8 caused more than 70% of the drug to be lost. However, the authors assumed that the acidic solution specifically has no effect on the container surfaces but this reduction in acidic solution possibly can be due to the domination of the polar monocationic form of the drug, which has good solubility in the hydroalcoholic solution.

It is possible that some treatments of the glassware could reduce the impact of the amount of loss due to adsorption. To increase the hydrophobicity or reduce the adsorption of PAHs onto the glassware, the use of silane coated (siliconised) glassware is considered particularly with a low concentration of solute, because the adsorption on these treated surfaces is weaker than on uncoated surfaces. The application includes introducing large molecules of a polymer of reactive silane such as chlorotrimethylsilane or dichlorodimethylsilane onto a piece of glassware (Seed, 2001; Qian et al., 2011). However, despite silanisation of glass surfaces adsorption of highly hydrophobic PAHs may still occur and the detailed mechanism is hard to clarify and requires further investigation (Qian et al., 2011). Another study conducted using silanised vials showed that the loss of oxycarboxin compound in these vials was reduced but not completely eliminated due to some active sites on the vial surfaces that remained even after treatment (Stanton, 1987).

4.2.3 Conclusion

HPLC-UV analysis was demonstrated to be a suitable and reliable method for the analysis of 1,4-DMN and 2-MeN and to assess the adsorption of these hydrophobic poly aromatic hydrocarbons from aqueous solutions. The HPLC chromatograms of aqueous standards showed peaks with good shape and stable retention time.

The peak areas showed no big difference between standards prepared in acetonitrile or in water. When preparing aqueous standards of 1,4-DMN from a stock solution in acetonitrile, analysis of samples after different periods of stirring showed that 2 – 3 hours mixing time with continuous stirring by a magnetic stirrer was sufficient to obtain good dissolution of 1,4-DMN. However, a mixing time of 24 hours was selected to ensure full dissolution.

The results of studying the stability of aqueous standard solutions at 1 µg/mL of 1,4-DMN and 2-MeN during ten days showed no significant change in the peak area over the time for both compounds in one replicate. A decline in the peak area for both compounds in another replicate may be due to bacterial degradation.

In this study, the precision of a mixed solution of two compounds in water at low concentration (0.1 µg/mL) showed acceptable values of RSD%. The linearity for the aqueous standard solutions was tested at different ranges of concentration (0.02 – 0.1 and 0.2 – 1.0 µg/mL). The coefficient of determination (R^2) values confirmed good linearity of the calibration curves. The LOD and LOQ of the studied compounds in aqueous solutions were calculated. Repeated injections led to lower and more plausible values than the calibration curve approach.

Adsorption of 1,4-DMN and 2-MeN to glassware surfaces was evaluated. The recovery from old glass containers with the exception of the soda glass screw top jars was found to be more than 89% for both compounds with acceptable precision. New volumetric flasks that had never been used before showed no adsorption. These glass materials are acceptable to use in future experiments and the slight loss due to adsorption can be controlled by applying the following cleaning procedure: Decon 90, 1 M NaOH, 1 M H₂SO₄ and ACN. On the other hand, the low recoveries of compounds when using plastic containers are unacceptable. Therefore using plastic containers should be avoided. In addition, adsorption onto the filters was found to be a big problem. Even though the Whatman filter paper No. 1 showed the best recovery this was only 80%.

During this stage of the project, it was unfortunate that the work in this investigation had to be suspended and not fully completed due to the global shortage and high cost of acetonitrile at the time of performing this work, supplies of acetonitrile did not return to normal level to complete this work. However, more investigation is required regarding the adsorption of 1,4-DMN onto the laboratory ware prior to quantitative determination of this sprout inhibitor in real water samples.

4.3 Chlorpropham and 3-chloroaniline

4.3.1 Materials and methods

4.3.1.1 HPLC system

The HPLC system used in this part of study is described in Section 2.1.2.

4.3.1.2 Chromatographic conditions

Analyses of CIPC and 3-CA were performed individually using the same chromatographic methods as described in Section 3.4.3.4. The exception was that 55% methanol was used as the mobile phase for analysis of 3-CA at a run time 10 minutes and with a retention time of ~ 6.5 minutes.

4.3.1.3 Preparation of standard solutions

For the preparation of stock aqueous solutions of 50 µg/mL of CIPC and 5000 µg/mL 3-CA in water, an accurate weight of 0.005 and 0.5 g respectively were weighed and dissolved in water in a 100 mL volumetric flask and made up to volume with water. The solutions were stirred for 24 hours using a magnetic stirrer in an incubator at 25 °C temperature for CIPC and 20 °C for 3-CA. Working solutions of 1 µg/mL were prepared from stock solutions of each compound and stored in the fridge at 4 °C.

4.3.1.4 Comparison of standards prepared in methanol and water

Standards prepared in water were compared with those in methanol by preparing five replicate standard solutions of 1 µg/mL of each of CIPC and 3-CA in each of water and methanol. A t-test was performed to check the difference between the two preparations.

4.3.1.5 Assessment of precision

To assess the precision of the analysis of CIPC and 3-CA standards in water, ten replicate injections of each standard solution of 1 µg/mL were carried out.

4.3.1.6 Linearity of the calibration curve for standard solutions

The linearity of the regression line of the calibration curve was evaluated by preparing two sets of standard solutions of each of CIPC and 3-CA in water at concentration ranges of 0.02 – 0.1 and 0.2 – 1.0 µg/mL. Each standard was injected twice.

4.3.1.7 Determination of the LOD and LOQ of CIPC and 3-CA in aqueous solution

The LOD and the LOQ of CIPC and 3-CA in water solution were calculated by two approaches as discussed in Section 2.1.4.3 by ten replicate injections of standard solutions of 1 µg/mL of each of CIPC and 3-CA and from the calibration curve at low range concentration (0.02 – 0.1 µg/mL).

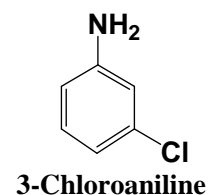
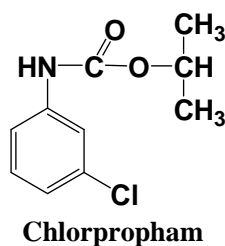
4.3.1.8 Examination of the recovery of CIPC and 3-CA using different laboratory ware

The adsorption of CIPC and 3-CA from aqueous solutions on different laboratory ware (glass, flask, plastic, filters and syringes) was examined.

4.3.2 Results and Discussion

4.3.2.1 Comparison of standards prepared in methanol and water

The polarity of the organic compounds plays an important role in their solubility in water. CIPC is a slightly polar compound whereas 3-CA tends to be highly polar forming strong hydrogen bonding with water. Therefore, the solubility of 3-CA in water is much higher than CIPC (see Figure 4:8).



Physical form	Crystal	Liquid
Molecular formula	C ₁₀ H ₁₂ ClNO ₂	C ₆ H ₆ ClN
Molecular weight (g/mole)	213.67	127.57
Water solubility (mg/L)	89 at 25 °C	5400 at 20 °C
Vapor pressure (mm Hg)	0.00018 at 20 °C	0.066 at 25 °C

Figure 4:8. Physiochemical data of chlorpropham and 3-chloroaniline (SRC, 2011).

Preparation of standard solutions of these compounds in water can be done in two ways: either through dissolving the primary material in water or by mixing the solutions in organic solvent. CIPC and 3-CA are much more soluble in water than 1,4-DMN and 2-MeN, therefore, they were directly dissolved in water.

The concentrations of stock solution were chosen to be below their solubilities in water. These solutions were noticed after a few minutes of mixing on the magnetic stirrer to be completely dissolved with the absence of any visible insoluble particles. However, to ensure complete dissolution of CIPC and 3-CA solutions they were mixed for 24 hours. This was verified by comparing standards of each compound prepared in aqueous solution with those in methanol. The chromatograms (see Figure 4:9) showed peaks of CIPC and 3-CA at the same retention time for both preparations with good shape of the CIPC peaks, however 3-CA chromatograms presented asymmetrical peaks having a little broader shape.

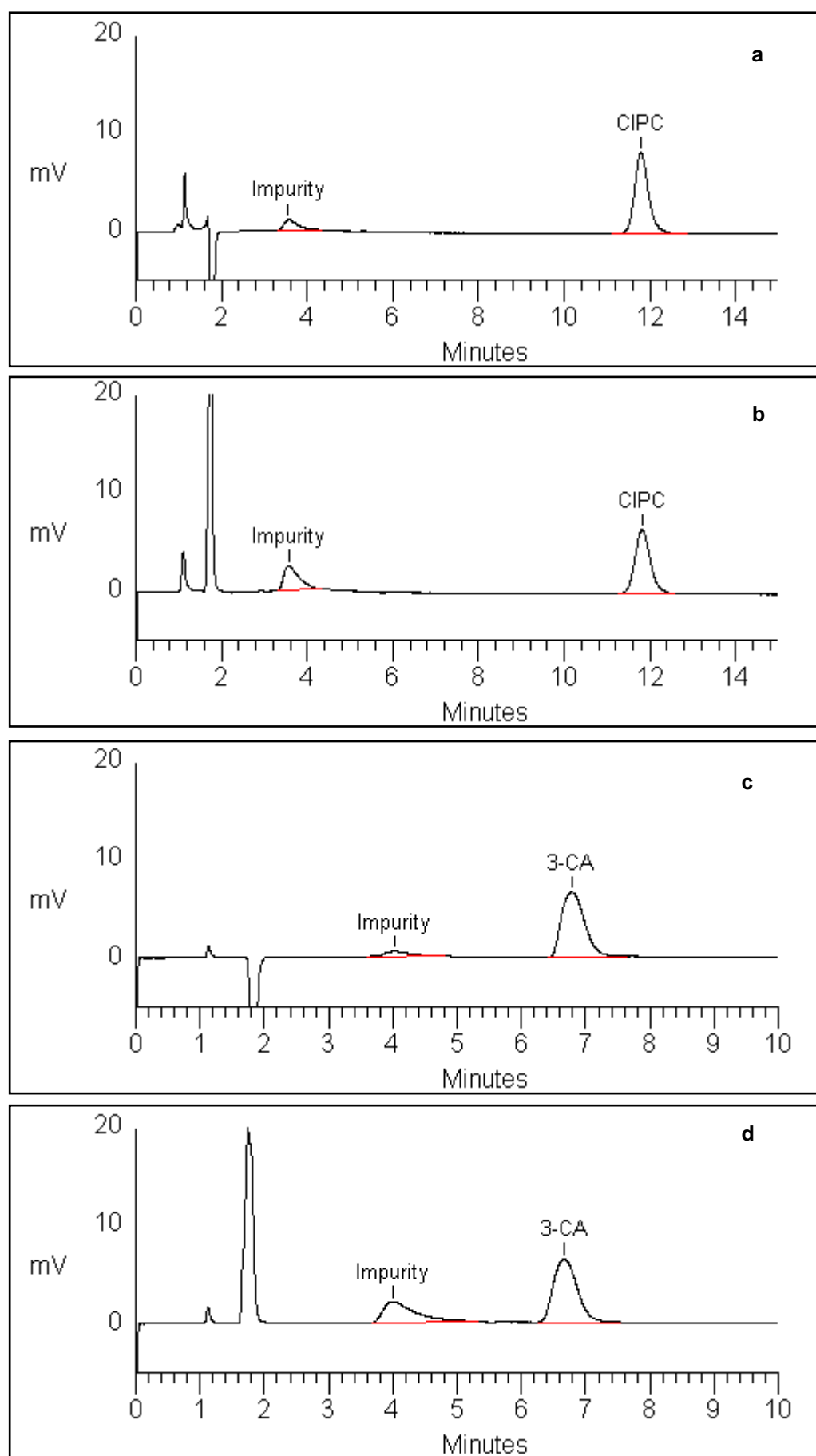


Figure 4:9. Chromatograms of 1 $\mu\text{g/mL}$ of solutions of: a- CIPC prepared in water, b- CIPC prepared in methanol, c- 3-CA prepared in water and d- 3-CA prepared in methanol.

From the chromatograms in Figure 4:9, it can be seen that the impurity peak in the water solution is a little smaller than with the methanol standards.

The results of a two sample t-test of the peak area for each method of preparation indicated a significant difference ($p = 0.00$) between the preparation of CIPC in water and methanol, that can be interpreted to be due to a weighing error during the preparation. In particular, a 50 $\mu\text{g/mL}$ stock solution of CIPC was prepared in water which can result in a larger weight error than preparing the highly concentrated solution in methanol (10 000 $\mu\text{g/mL}$). No significant difference was found between the preparation of 3-CA in water and its preparation in methanol.

Table 4:7. The mean peak area and results of the t-test for each compound prepared as 1 $\mu\text{g/mL}$ solutions of methanol and water.

Compound	Mean peak area (n = 5)		t-test
	Methanol	Water	
CIPC	16362053	19193052	S*
3-CA	16215163	16132725	NS*

S*: significant difference ($p < 0.05$), NS*: no significant difference ($p > 0.05$)

4.3.2.2 Assessment of precision

The precision of the standards in aqueous solution was measured. Using ten replicate injections of 1 $\mu\text{g/mL}$ of each aqueous solution of CIPC and 3-CA showed good precision with RSD% values 0.8% and 0.3% respectively. Thus, the precision of the standard preparations in water can be considered acceptable based on the precision criteria previously discussed (see Section 3.2.3.2).

4.3.2.3 Linearity of the calibration curve for standard solutions

The regression line was plotted between the peak areas of each compound against the corresponding concentrations for two ranges (0.02 – 0.1 and 0.2 – 1.0 $\mu\text{g/mL}$) as shown in Figures 4:10 and 4:11. Good linearity was assessed according to the coefficient of determination (R^2) which gave values ≥ 0.993 , which is greater than R^2 selected for the linearity criteria (0.990) in this study (see Section 3.2.3.3).

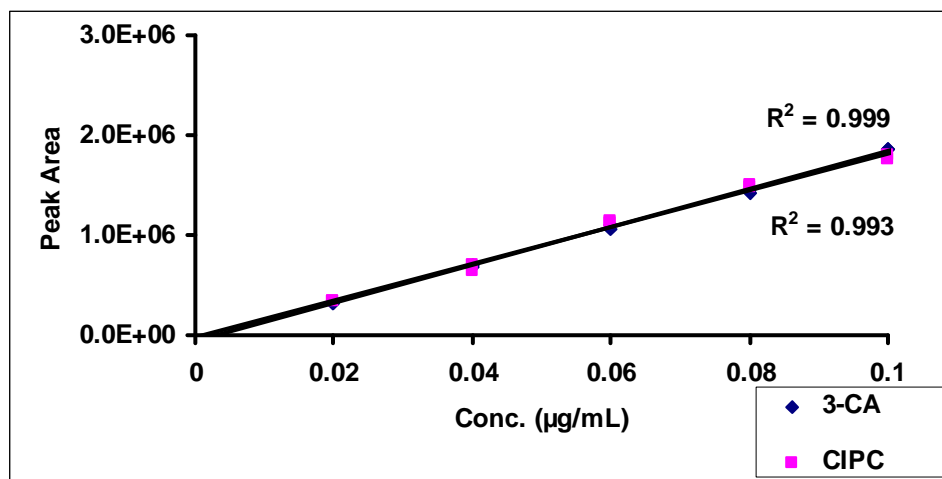


Figure 4:10. Calibration graph for 3-CA and CIPC at a range of between 0.02 and 0.1 µg/mL in aqueous solution.

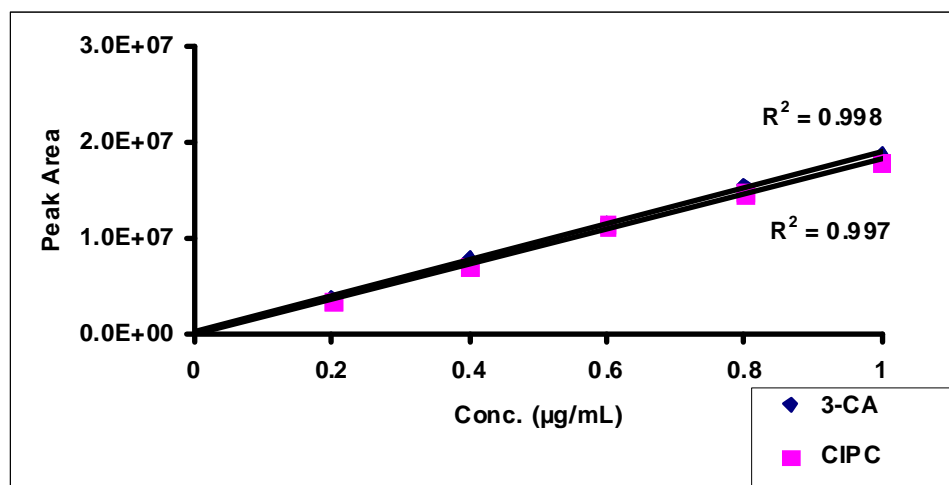


Figure 4:11. Calibration graph for 3-CA and CIPC at a range of between 0.2 and 1.0 µg/mL in aqueous solution.

4.3.2.4 Determination of the LOD and LOQ of CIPC and 3-CA in aqueous solution

The LOD and LOQ of ten replicate injections of aqueous solutions of 1 µg/mL of each of CIPC and 3-CA presented higher values than the calibration curve approach (see Table 4:8). The reason might be due to the high concentration chosen. In contrast, the calibration curve approach proved to give reliable and more practical values of these compounds in aqueous solution. It should also be pointed out that these values are close to those obtained in methanol solutions that were discussed previously in Section 3.4.3.7.

Table 4:8. LOD and LOQ values based on the repeatability injections (n = 10) of 1 µg/mL of CIPC and 3-CA and the statistical data for the calibration curve in the range between 0.02 and 0.1 µg/mL.

Assessed Approach	LOD (µg/mL)		LOQ (µg/mL)	
	CIPC	3-CA	CIPC	3-CA
Injection repeatability (1 µg/mL)	0.024	0.009	0.081	0.030
Calibration curve (0.02 – 0.1 µg/mL)	0.008	0.003	0.026	0.009

4.3.2.5 Examination of the recovery of CIPC and 3-CA using different laboratory ware

The adsorption of CIPC and 3-CA onto laboratory glassware in aqueous solutions was investigated. The recovery of compounds in their aqueous solution was measured after contact with surfaces of laboratory ware that are commonly used in the quantitative analysis such as glass, plastic, filters and syringes.

The results in Tables 4:9, 4:10 and 4:11 show excellent recovery of 3-CA from its solution and very low adsorption of CIPC. However, some plastic materials (HDPE bottle and PALL Acrodisc syringe filter) caused more adsorption of CIPC.

High recoveries of 3-CA were obtained due to its higher polarity and solubility that provided a strong interaction with water allowing it to remain in the aqueous solution. However, the loss of CIPC might be caused due to its lower polarity. The low recovery of CIPC resulted from the syringe filter (PALL Acrodisc 13 mm) may be interpreted as due to chemical incompatibility.

As a conclusion, bottles and filters with acceptably low adsorption can be selected from those tested for future experimental work.

Table 4:9. The recovery% of CIPC and 3-CA using different glass containers.

Glass containers	Material	CIPC		3-CA	
		Recovery	RSD%	Recovery	RSD%
		%	(n = 5)	%	(n = 5)
Volumetric flask (10 ml)	Borosilicate	97	0.9	101	0.2
Quick fit conical flask (25 ml)	Borosilicate	98	1.8	100	0.8
Screw top jar (100 ml)	Soda	97	1.4	100	0.3

Table 4:10. The recovery% of CIPC and 3-CA using different plastic materials.

Plastic containers	CIPC		3-CA	
	Recovery	RSD%	Recovery	RSD%
	%	(n = 5)	%	(n = 5)
Bottle Polypropylene	96	1.2	100	0.9
Bottle HDPE	92	1.5	100	1.0
Plastic syringe (BD Plastipak)	97	0.8	99	0.4
Micro pipettor tip	97	1.9	100	0.3

Table 4:11. The recovery % of CIPC and 3-CA using syringe and filters.

Filters specification	CIPC		3-CA	
	Recovery	RSD%	Recovery	RSD%
	%	(n = 5)	%	(n = 5)
Chromacol syringe filter (PTFE)	93	1.2	99	1.3
PALL Acrodisc syringe filter (supor)	61	9.9	93	0.5
Glass microfiber filter paper 47 mm	97	0.9	99	0.9

4.3.3 Conclusion

Standard solutions of CIPC and 3-CA in water were prepared and compared with those prepared in methanol. Standard solutions of 3-CA showed no significant difference when prepared in water or methanol, whereas a significant difference with CIPC preparations was reported due to the difficulty in weighing the small weight needed to prepare the stock solution of CIPC.

Good precision was determined for ten replicate injections of 1 µg/mL of each aqueous solution of CIPC and 3-CA with RSD% values of less than 1. Good linearity was found according to the coefficient of determination (R^2) values of ≥ 0.993 . The LOD and LOQ measurements showed lower values by calculating the standard deviation based on the calibration curve approach compared to replicate injections. These values were found to be close to those obtained for the methanol solution preparations.

The possibility of adsorption of CIPC and 3-CA onto glassware in aqueous solution was studied. The experimental recoveries indicated excellent recovery of 3-CA with all types of laboratory ware tested. CIPC showed recoveries greater than 92% with most of the materials tested. In general, these results are acceptable and caused no great concern for the adsorption of these compounds onto laboratory ware; therefore these materials can be used for the quantitative analysis of CIPC and 3-CA in water samples.

The adsorption of 3-CA onto laboratory ware would also be important to investigate and evaluate the factors related to bind 3-CA onto potato peel surfaces; this will be discussed in Chapter 6.

Chapter 5: Extraction method for the determination of CIPC and preliminary analysis of its metabolite 3-CA in potato samples

5.1 Introduction

After application of sprout inhibitors to potatoes in stores, residues or degradation products remaining in the potato tubers are of concern for consumers due to their possible toxicity. Therefore, determination of their levels in potatoes is very important for the potato processing industry and human consumption. Studies that have been undertaken to measure the residues of these sprout inhibitors showed that the majority of the residue remaining is from the parent pesticides but some metabolites have also been found in potatoes treated with sprout suppressants (FAO and WHO, 2001; Orejuela and Silva, 2005).

Residues of chlorpropham and its metabolite 3-CA have been identified in treated potatoes after long term storage (Orejuela and Silva, 2005; Worobey and Sun, 1987; FAO and WHO, 2001; McGowan et al., 2010). Nowadays, the determination of CIPC and its metabolite 3-CA in potato samples is receiving increasing attention by the potato industry. The MRL of CIPC should include both CIPC and 3-CA. Thus, the focus has been towards developing reliable and rapid methods to extract and quantify these residues in potato samples.

A number of extraction techniques have been employed to extract CIPC residues from potatoes and other matrices. Conventionally, simple solvent extraction using solvents such as methanol, acetone, hexane and methylene chloride coupled with GC analysis or HPLC has been widely used. Homogenisation is also one of the methods commonly employed to extract CIPC residue from potato samples by blending with an organic solvent (Lentza-Rizos and Balokas, 2001; Tsumurahasegawa et al., 1992; Nagami, 1997). Most recently, numerous papers have reported the successful use of new techniques of extraction for CIPC. Two optimized methods with accelerated solvent extraction (ASE) and Soxhlet for extraction of chlorpropham from potatoes were presented (Schuermann et al., 2006). Additionally, Solid Phase Micro-Extraction (SPME) followed by GC/MS analysis have been applied to extract CIPC in potatoes (Volante et al., 1998). The extraction procedure involved homogenising a potato sample which was diluted in water to create a suspension

and extracted with a 100 μm thick polydimethylsiloxane fiber then desorbed into the injection port of the GC-MS. The residue results of this method in potatoes corresponded to those obtained with a traditional multiresidue method. Ultrasonic solvent extraction coupled with thin-layer chromatography was reported by Babic and co-workers (1998) to extract CIPC from soil. The extraction method was optimised regarding the volume of solvent, the optimum time of sonication and number of extraction steps. This method showed good extraction efficiency combined with simplicity of use and the solvent consumption was significantly lower. Sun and Lee (2003) made a comparison between microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) using HPLC with UV detection to extract CIPC from soil, SFE exhibited slightly higher recovery for CIPC than MAE. Although these techniques are less time consuming and have low solvent consumption the apparatus has high cost which can only be justified when analysing large numbers of samples.

In reviewing the literature, no suitable validated method was found and specified for the associated determination of the parent chlorpropham and its metabolite 3-CA in commercial potatoes by HPLC analysis. However, analytical methods have been reported to determine CIPC alone or combined with different metabolites.

Some methods have been used with varying success applying various types of solvents and analytical techniques. Beernaert and Hucorne (1991) developed a simple and rapid method for the quantitative determination of residual CIPC and IPC in fresh potatoes. The potato was cut into small pieces and mixed with water to obtain a homogenous slurry, which was extracted by adding methylene chloride. After centrifuging and concentrating, the extract was transferred to a 2 mL calibrated tube containing 2-chloroaniline as an internal standard then made to volume with hexane prior to quantitative analysis by GC. The recovery results at spiking levels of 0.5, 1.0 and 5.0 mg/kg were $99 \pm 10\%$ and $100 \pm 15\%$ for CIPC and IPC respectively. The limit of detection for both compounds was 0.1 mg/kg. Analysis of 161 potato samples using this method reported that 18 samples exceeded the maximum tolerated value of 5 mg/kg (which was established in Belgium by Royal Decree in 1988). The maximum residue found for CIPC was 15.4 mg/kg (Beernaert and Hucorne, 1991).

The residue of CIPC has been extracted from crisps by a method involving solvent extraction by blending, clean up with an alumina column and GC analysis. This method reported residue levels of CIPC in potato slices prior to frying, crisp samples immediately after frying and fryer oil to be 0.18, 0.45 and 0.4 mg/kg respectively. Crisps produced from

untreated potato were spiked with 100 µg CIPC in hexane solution, after allowing the solvent to evaporate, the crisps were extracted and the total recovery rate was found to be 93.2%; the minimum detectable amount of CIPC was 0.035 mg/kg (Ritchie et al., 1983).

Worobey and Sun (1987) analysed the potato peel of potatoes samples taken from different supermarkets to determine the residue levels of CIPC and two of its degradation products; 3-CA and 3,3 –dichloroazobenzene (3,3-DCAB). Potato tubers were washed to remove any particles of soil and peeled, taking 20 g from the peel for analysis with 50 mL of methanol and homogenising in Polytron blender for 4 minutes. The macerate was filtered and dried over anhydrous sodium sulphate then rotary evaporated under vacuum. The methanol extract was combined with saturated NaCl solution and partitioned into methylene chloride. After washing the methylene chloride extract with further saturated NaCl, trimethyl pentane (TMP) was added and used to transfer the analytes by rotary evaporation of methylene chloride. Finally, the TMP extract was analysed using gas chromatography coupled with electron capture detection (GC-ECD) and gas chromatography coupled with mass spectrometry (GC-MS). The chromatograms of both the analyses of the extract of potato peel showed peaks of 3-CA, CIPC and 3,3-DCAB. The residue level for duplicate injections of several extracted potato samples ranged from 21 – 166 µg/kg (CIPC), 0.18 – 0.36 µg/kg (3-CA) and 2 – 39 µg/kg (3,3-DCAB). The authors interpreted the formation of both metabolites to hydrolysis of CIPC to 3-CA which transformed to 3,3-DCAB through peroxide oxidation or diazotisation reduction and coupling. Another assumption was that 3-CA occurred as a contamination in the formulation, since CIPC is synthesised commercially through reacting 3-CA with isopropylchloroformate. Recovery results by this method were 87.5% for CIPC (at spiking level 20 µg/kg), 6.3% for 3-CA (2 µg/kg) and 59% for 3,3-DCAB (2 µg/kg). However, no explanation was offered for the low recovery of 3-CA in this method.

Coxon and Filmer (1985) treated two varieties of potatoes with various concentrations of ^{14}C or ^{36}Cl -CIPC and stored them for 6 months at 10 °C in a 5 L flange flask under controlled ventilation conditions in the laboratory. For the extraction of CIPC residues and identification of its metabolites, the peel was immersed in boiling methanol for 20 minutes and cooled before homogenisation blending. The methanol extract after filtration and clean up process was analysed and only CIPC was found. There was no evidence of 3-CA or any other degradation product of CIPC in the peel extract, although there was 27.4 – 29.2% of the radioactivity label found as non-extractable bound residues in peel (Coxon and Filmer, 1985).

Chlorpropham and propham in potatoes were determined by Orejuela and Silva (2004) using HPLC with peroxyoxalate chemiluminescence (PO-CL). After decarboxylation of IPC and CIPC by basic hydrolysis to aniline and 3-CA respectively they were readily derivatised with dansyl chloride for a short time and the dansylated amines were analysed by HPLC achieving good separation with an RP C₁₈ column and 60% aqueous acetonitrile solution as the mobile phase at a flow rate 0.8 mL/min. The recovery results from spiking potato samples with CIPC and IPC at 500 µg/kg ranged from 97.5% to 103.2% using dichloromethane as the extractant in the presence of saturated sodium chloride. The reliability of this method was assessed by validating the sensitivity, linearity, limit of detection and precision. The limit of detection was reported as 3.5 µg/kg. The choice of applying this rapid and sensitive method is useful for the determination of CIPC and IPC, however, no attempt was made to deal with 3-CA (Orejuela and Silva, 2004).

Orejuela and Silva (2005) also developed an analytical method for the multi-residue analysis of CIPC and aniline metabolites namely 3-chloroaniline, 3-chloro-4-hydroxyaniline and 3-chloro-4-methoxyaniline in potato samples. The method involved a derivatisation procedure of a mixed aqueous solution of the analytes with 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein (DTAF) as a fluorescence agent (since the analytes are not fluorescent), then using micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (MEKC-LIF) for separation and determination. Potatoes were chopped with a food processor then subsamples were spiked with aniline metabolites or CIPC prior to homogenisation and extraction with dichloromethane. The recovery results for the aniline metabolites at spiking levels of 10 – 250 µg/kg were over 97%. Although this method determines the parent pesticide CIPC and aniline metabolites the drawback is the long laboratory procedure that requires a derivatisation step (Orejuela and Silva, 2005).

Several unpublished methods were reviewed by the Joint Meeting on Pesticide Residues (JMPR) in 2001 for the determination of residues of CIPC alone or of the parent and three metabolites namely; 3-CA, 4-hydroxy-CIPC and para-methoxy-CIPC in potatoes. Most of these methods involved homogenisation and extraction with an organic solvent (e.g. methanol, petroleum ether/acetone, hexane/acetone) followed by partition into dichloromethane. For further purification, a Florisil column was used. Following transfer into a volatile solvent, determination was carried out using gas liquid chromatographic coupled with nitrogen phosphorus detection (GLC-NPD) or by gas liquid chromatography coupled with electron capture detection (GLC-ECD) after bromination. Three methods

were described in detail and the recovery data from spiking whole potato and fresh peel was found to be quite variable ranging from 36 – 128% for CIPC, 51 – 120% for 4-hydroxy-CIPC, 72 – 129% for para-methoxy-CIPC and from 0 – 77% for 3-CA. Several samples were with recoveries outside acceptable 70 – 120% range. Only one method quoted the LOD and LOQ values to be 0.08 and 0.45 mg/kg respectively in whole potatoes, fresh pulp and peel and processed wet peel (FAO and WHO, 2001).

Methanol extractant and HPLC analysis were used by Wilson et al., (1981) to extract the residue of CIPC from spiked potatoes and three other foodstuffs, peas, beans and blueberries. Potatoes were spiked at levels ranging from 0.25 – 81 mg/L and the extract was cleaned up using an acid aluminium column prior to HPLC analysis to give recoveries in the range 64 – 102% for all four foods. The results showed that recovery of 100% or better was obtained at higher concentrations (above 1 mg/L of CIPC) while the recovery was less at lower concentrations. The limit of the detection was 0.12 mg/kg. However, this method was not applied for determining CIPC metabolites (Wilson et al., 1981).

In recent decades, many researchers have developed methods to extract CIPC from potatoes at the University of Glasgow laboratories. Boyd (1988) developed an extraction method by blending the whole chopped potato with hexane for 1 minute at high speed in an electric blender in the presence of anhydrous sodium sulphate. The homogenised mixture was quantitatively transferred to an aluminum bottle with hexane and shaken for 30 minutes and left 24 hours prior to filtration. The residue of the filtration was washed through a filter paper with hexane many times and then the filtrate extract obtained was concentrated using a rotary evaporator prior to analysis by GC (Boyd, 1988).

Baloch (1999) developed a method based on Soxhlet extraction by chopping the whole potato tuber with an electric food processor. A subsample was taken from the homogenised chopped sample and placed into a cellulose thimble with anhydrous sodium sulphate then extracted with hexane for two hours in a Soxhlet extracting unit. The extract was evaporated to dryness using a rotary evaporator at a temperature below 40 °C and then 2 mL of hexane was added to the flask to redissolve the evaporated extract, which was transferred and then loaded into the GC. This method was found to be easier and quicker than the blending procedure by Boyd (1988). Currently, the Baloch (1999) method with some modifications is used as the routine method at the laboratory of University of Glasgow applied to treated commercial potato samples.

Since previous studies have shown that most CIPC residue is mainly found to be in the outer layers (approximately 2 mm thickness) of treated potato tubers with little or no residue in the pith (Singh et al., 2009; Corsini et al., 1979; Lentza-Rizos and Balokas, 2001; Coxon and Filmer, 1985; Worobey and Sun, 1987; Mondy et al., 1992a; Worobey et al., 1987), emphasis is placed upon extraction and determination of CIPC in the potato peel, which represents the CIPC in whole potato tubers. For that reason, the Baloch (1999) method was modified taking potato peel instead of whole potato tuber to extract CIPC (Geraldine McGowan, personal communication).

However, all the previously mentioned methods suffer from some disadvantages related to cost, large solvent consumption and long laboratory procedures which restrict the number of potato samples that can be analysed per day. Therefore, a simple and rapid analysis is required in terms of less solvent use, equipment and analytical steps.

Recently, a simple extraction procedure for peel samples involving a small volume of acetonitrile solvent coupled with HPLC analysis has been carried out by researchers at the University of Glasgow for the determination of CIPC residues in potatoes (Khan et al., 2008). The method comprised extracting of a representative subsample of 5 g of potato peel in 40 mL of acetonitrile in a 100 mL glass bottle left overnight at room temperature. The extract was filtered through a 0.2 µm PTFE membrane syringe filter and analysed using HPLC coupled with UV detection.

This soaking method was validated by comparison with the Baloch (1999) method showing good correlation but with a 25% greater residue of CIPC obtained by the soaking method. The LOQ for the HPLC analysis based on a spiked extract was 0.01 mg/kg in potato tubers. The recovery result by spiking peel was found to be 94% at spiking level of 2 mg/L. Currently, this method is also used at the University of Glasgow to analyse potato samples treated with CIPC. The main advantages of this method are:

- Simple, faster analysis and fewer steps in the laboratory procedure.
- The extract is more concentrated.
- Less solvent is used.
- No need to rotary evaporate.

- Greater sensitivity (lower LOQ) when coupled with HPLC.
- Inexpensive and applicable to a wide range of potato samples on a daily basis.
- Low risk of CIPC evaporation.
- Satisfies safety requirements.

However, due to the acetonitrile shortage, it was essential to develop and validate an analytical method for the determination of CIPC using an alternative extracting solvent. Moreover, the methods used at the University of Glasgow have been focussed only on the determination of CIPC not its metabolites such as 3-CA and therefore optimised analytical methodologies are required for both CIPC and 3-CA.

The efficiency of extraction of potato peel using a small volume of solvent relies on many factors mainly the polarity of the solvent used, peel surface area, the contact between the peel and the solvent, temperature and agitation or shaking. Conventional solvent extraction systems include methanol and acetonitrile. Therefore, the main objectives of the work in this chapter were to:

- Develop a method to extract and analyse both CIPC and its metabolite 3-CA using methanol to overcome the problem of acetonitrile supply at the time this study carried out.
- Validate the new method through a recovery study of CIPC and its metabolite 3-CA by spiking potato samples at different spiking levels.
- Calculate the LOD and the LOQ for CIPC and 3-CA with IPC as internal standard using this method.
- Correlate the new method with the routine method of hexane Soxhlet extraction coupled with GC analysis which is routinely used at the University of Glasgow laboratory to extract and analyse residues of CIPC in potato samples.

5.2 Materials and Methods

5.2.1 Methods

5.2.1.1 Standards

See Section 2.1.1 of preparation of standard solutions of CIPC, IPC and 3-CA in methanol and hexane.

5.2.1.2 HPLC analysis

The HPLC system used is described in Section 2.1.2 and the chromatographic conditions for the HPLC analysis method are summarised in Section 3.4.3.4

5.2.1.3 GC analysis

See Section 2.1.7 for GC analysis system and chromatographic conditions.

5.2.1.4 Methanol soaking extraction

The soaking extraction procedure involved peeling the potato, chopping the peel into fine pieces and mixing to obtain a homogenous sample (see Section 2.1.5). 5 g of chopped peel sample from the potato peel tuber was weighed into a 100 mL screw top jar (as no adsorption of CIPC and 3-CA onto this container as shown in Table 4:9), then 40 mL methanol containing the internal standard of 10 µg/mL protham (IPC) was added as the extracting solution. The samples are left soaking overnight (~ 16 hours) at room temperature. The extract was filtered and transferred into HPLC vials through a 0.2 µm PTFE (Teflon) membrane syringe filter and analysed twice. The standard solution was a mixed solution of 10 µg/mL of 3-CA, IPC and CIPC prepared in methanol (injected in duplicate).

The residue concentration of CIPC and 3-CA in the extract and whole potato tuber was calculated as follows:

$$\text{Conc. in extract (}\mu\text{g/mL)} = \frac{[\text{PA in sample} * \text{Conc. of Std} * \text{PA IS in Std}]}{[\text{PA in Std} * \text{PA IS in sample}]}$$

$$\text{Conc. in tuber (mg/kg)} = \frac{[\text{Conc. in extract} * \text{Vol. of extract (40 mL)} * \text{Wt of total peel}]}{[\text{Wt of peel sample} * \text{Wt of potato tuber}]}$$

The recovery of CIPC, IPC and 3-CA was calculated as follows:

$$\text{Recovery\%} = \frac{[\text{Conc. in extract } (\mu\text{g/mL})]}{[\text{Conc. in Std } (\mu\text{g/mL})]} * 100$$

Note:

Conc.: concentration

PA: peak area

PA IS: peak area of internal standard

Std: standard solution

Vol.: volume

Wt: weight

It should be pointed out that the internal standard of IPC was used to minimise the analytical error due to dilution of the extracted compounds caused by the presence of water in the potato peel. Principally, the percentage of water represents approximately 90% of the potato peel weight (see Section 2.1.9). In this study, IPC was selected as the internal standard owing to its similar structure of CIPC, but with the absence of a single chlorine atom.

5.2.1.5 Hexane Soxhlet extraction

The Soxhlet extraction procedure reported by Baloch (1999) was applied to extract CIPC from treated potatoes. The remainder of the peel from each tuber (left from the methanol soaking extraction) was placed into a Soxhlet apparatus for extraction as described in Section 2.1.6. The extract in the round bottom flask was quantitatively transferred to a 100 mL volumetric flask and made up to volume. The extract was divided into two portions (each 50 mL) for simultaneous analysis by HPLC and GC. For HPLC analysis, the sample was filtered and analysed as described in Section 5.2.1.4. While the other 50 mL was concentrated using a rotary evaporator system at 35 °C to obtain 1 mL CIPC extract. Then 200 µL of the 1000 µg/mL propham (IPC) internal standard was added and the volume was made up to 2 mL for analysis by GC.

The concentration of CIPC and 3-CA residues in each tuber was calculated according to the extraction method and technique used is as follows:

Hexane-Soxhlet- HPLC

$$\text{Conc. in extract } (\mu\text{g/mL}) = \frac{[\text{PA in sample} * \text{Conc. of Std}]}{[\text{PA in Std}]}$$

$$\text{Conc. in tuber (mg/kg)} = \frac{[\text{Conc. in extract} * \text{Vol. of extract (100 mL)} * \text{Wt of total peel}]}{[\text{Wt of peel sample} * \text{Wt of potato tuber}]}$$

Hexane-Soxhlet- GC

$$\text{Conc. in tuber (mg/kg)} = \frac{[\text{Conc. in extract} * \text{Vol. of extract (100 mL)} * \text{Wt of total peel}]}{[\text{Wt of peel sample} * \text{Wt of potato tuber}] * 25}$$

Note: the number 25 refers to the concentration factor from 50 mL extract to 2 mL of the concentrated extract.

5.2.2 Comparison of standard solutions prepared in organic potato extract and in methanol

Organic potatoes untreated with any pesticide were purchased from a local supermarket. An extract of organic potato peel was obtained by soaking 5 g peel ($n = 15$) overnight with 40 mL of methanol. The extracts were pooled and filtered under vacuum through a glass microfiber filter (GF/C, 47 mm) joined with a supor membrane filter (0.2 μm 47 mm). After collection of the filtrate, three replicates of a mixed spiked solution of CIPC, IPC and 3-CA were prepared at concentrations 0.1, 1 and 10 $\mu\text{g/mL}$. Standard solutions of the same number of replicates and concentrations were also prepared in methanol. These solutions were injected in duplicate into the HPLC system to compare the standards in organic potato extract and methanol.

5.2.3 Detection limit of the studied compounds in the organic potato extract

The LOD and LOQ for CIPC, IPC and 3-CA in potato extract were estimated by replicate injections ($n = 10$) of a 0.05 $\mu\text{g/mL}$ mixture of CIPC, IPC and 3-CA prepared in an extract of organic potato.

5.2.4 Assessment of the recoveries of CIPC, IPC and 3-CA from spiking organic potato peel

The methanol-soaking-HPLC method was applied to measure the recovery of CIPC, IPC and 3-CA. 5 g of organic potato peel was spiked with 200 μ L of spiking solution of mixed CIPC and 3-CA at three concentrations, namely 0.1, 1.0 and 10 μ g/mL (5 replicates) as shown in Table 5:1. The bottles were sealed for 1 hour prior to extraction with 40 mL of methanol (containing IPC as the internal standard) for approximately 16 hours (see Section 5.2.1.4). Additionally, 5 replicates of a control with no peel were carried out when 200 μ L of spiking solution was added directly to empty bottles which were sealed for 1 hour prior to carrying out the same extraction.

Table 5:1. The spiking levels and extract concentrations for extracting 5 g of organic potato peel.

Wt. of Peel	Spiking solution (CIPC + 3-CA)	Extracting solution (IPC)	Spiking level in peel	Conc. in extract
5 g	200 μ L 20 μ g/mL	40 mL 0.1 μ g/mL	0.8 μ g/g	0.1 μ g/mL
5 g	200 μ L 200 μ g/mL	40 mL 1 μ g/mL	8.0 μ g/g	1.0 μ g/mL
5 g	200 μ L 2000 μ g/mL	40 mL 10 μ g/mL	80 μ g/g	10 μ g/mL

5.2.5 Variability of CIPC residues and uniformity of a mixed peel sample

To evaluate the effect of the uniformity of the peel sample on the variability of CIPC residue measurement in treated potatoes, three potatoes treated with CIPC were peeled and the peel was chopped into small pieces and well mixed. Ten replicates from the pooled peel were extracted and analysed by HPLC (see Section 5.2.1.4). The variability is expressed by the RSD% of the residue of CIPC.

5.2.6 Final validation of the methanol soaking-HPLC method

To prove the applicability and reliability of the methanol-Soaking-HPLC method, it was compared to the hexane Soxhlet-GC method (which is the standard method used at the University of Glasgow).

5.2.6.1 Correlation between the developed method and the hexane Soxhlet–GC method for residue analysis of CIPC

Randomly, 29 individual potato tubers which had been treated with CIPC were chosen from large commercial stores. After washing and drying procedures were performed (as described in Section 2.1.5), 5 g from the peel of each tuber was extracted and analysed by the methanol-soaking-HPLC method (see Section 5.2.1.4) leaving the remainder of the peel for hexane-Soxhlet-GC analysis (see Section 5.2.1.5). The extract from the Soxhlet extraction was also analysed by HPLC to compare between soaking extraction and Soxhlet extraction.

To compare standards prepared in methanol and hexane, five replicate solutions of the same concentration (1 µg/mL) of a mixture of CIPC, IPC and 3-CA were prepared in each of methanol and hexane. These solutions were injected in duplicate into the HPLC system.

5.2.6.2 Determination of 3-CA in commercial potato samples treated with CIPC

The experiment in Section 5.2.6.1 was extended by measuring the residues of 3-CA in the 29 potato tubers by application of the methanol soaking-HPLC method.

5.3 Results and Discussion

5.3.1 Comparison of standard solutions prepared in organic potato extract and in methanol

In order to obtain the extract of potato peel, fresh organic potato peel was soaked overnight in methanol. After collecting the extract filtrate, samples were analysed and compared with standard solutions of 1 µg/mL of solution of CIPC, IPC and 3-CA prepared in methanol. Standards of the three compounds were also prepared in a pooled extract of organic peel and compared with standards prepared in methanol at three concentrations (0.1, 1.0 and 10 µg/mL) using t-tests. Figure 5:1 compares the chromatograms obtained from HPLC analysis of the extracts and standards.

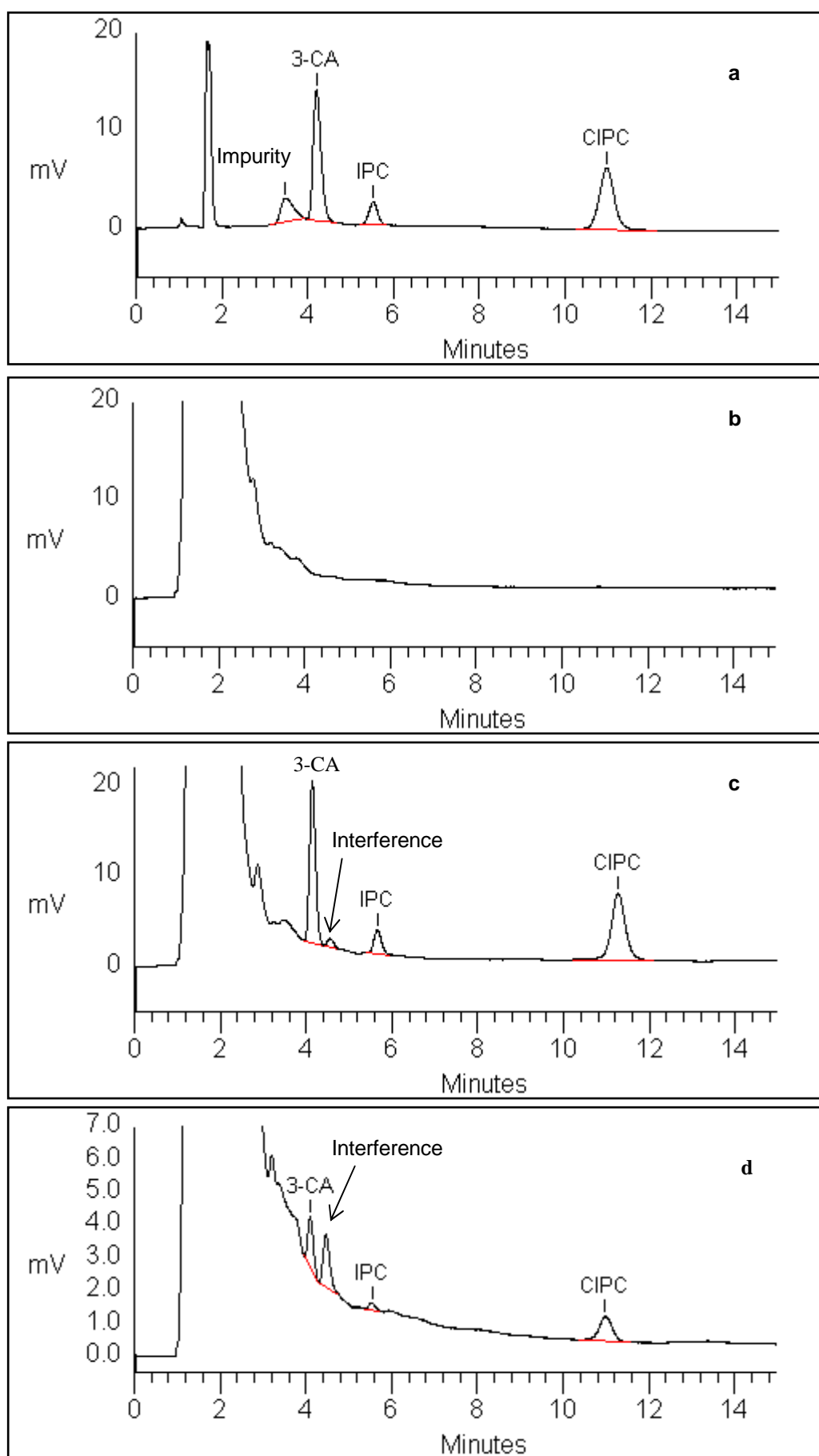


Figure 5:1. Chromatograms of a- 1 µg/mL solution of CIPC, IPC and 3-CA prepared in methanol, b- extract of organic potato peel, c- 1 µg/mL solution of CIPC, IPC and 3-CA prepared in an extract of organic potato peel and d- 0.1 µg/mL solution of CIPC, IPC and 3-CA prepared in extract of organic potato peel.

As can be seen from Figure 5:1 b, HPLC analysis of the extract of organic potato peel produced a typical chromatogram showing overlapping peaks of co-extracted compounds which eluted earlier in the chromatogram according to their polarity. Potato peel contains moisture, crude fat, crude protein, ash, crude fibre and carbohydrate (Mohdaly et al., 2010; Camire et al., 1997; Shukla and Kar, 2006). Extraction of potato peel in an organic solvent yields an extract containing compounds such as flavonoids, phenolic compounds, anthocyanins and glycoalkaloids (Mohdaly et al., 2010; Al-Weshahy and Rao, 2009; Ponnampalam and Mondy, 1983). The solvent used plays an important role during extraction of plant material. Commonly, highly polar solvents particularly methanol, ethanol and acetone show good ability to extract materials (e.g. phenolic compounds, flavonoids) from potato peel compared with lower polarity solvents such as hexane, diethyl ether and petroleum ether (Mohdaly et al., 2010).

On comparison with the standard solution of CIPC, IPC and 3-CA in methanol, the extract chromatogram in Figure 5:1 b shows a good and clean baseline, free from interfering peaks in the region of the retention times of the three compounds. Thus, no further clean up step is required for the extraction procedure and analysis, saving time, effort and cost. The non-appearance of CIPC and related compounds in the extract confirmed that the organic potatoes had not received any contamination from CIPC.

The Figure 5:1 also compares the chromatograms of mixed standard solutions of CIPC, IPC and 3-CA prepared in both methanol and the organic potato peel extract. In the spiked peel extract, co-extractive interference peaks appeared close to the 3-CA peak but the peaks were well resolved and no effect of interference of co-extractive materials was seen. All the chromatograms produced peaks at the same retention times for 3-CA, IPC and CIPC peaks (approximately 4.5, 5.5 and 11 minutes respectively).

Comparison between the two matrices at three concentration levels (0.1, 1.0 and 10 $\mu\text{g/mL}$) was made by a paired t-test of the peak areas using Minitab as shown in Table 5:2.

Table 5:2. Paired t-test for the preparation of mixed standards CIPC, IPC and 3-CA in organic potato peel extract and methanol at varying concentrations.

Compound	t-test (p-value)		
	0.1 µg/mL (n = 6)	1 µg/mL (n = 6)	10 µg/mL (n = 6)
CIPC	NS* (p = 0.465)	S* (p = 0.016)	NS (p = 0.088)
IPC	NS (p = 0.352)	NS (p = 0.667)	NS (p = 0.156)
3-CA	NS (p = 0.268)	NS (p = 0.836)	NS (p = 0.466)

NS*: no significant difference ($p > 0.05$), S*: significant difference ($p < 0.05$)

It is apparent from the table that the results show no significant differences ($p > 0.05$) between matrices for all compounds at each of the studied concentrations with the exception of the preparation of CIPC at 1 µg/mL which did indicate a significant difference. The reason for this difference is not clear but it may be attributed to contamination or volumetric errors.

In conclusion, methanol extracts were found to be suitable for HPLC separation of CIPC, IPC and 3-CA and can be used for further work. No interfering peaks were found at the retention times of the three compounds. However, consideration should be taken to avoid matrix interference from the peel extract early in the chromatogram close to the 3-CA peak particularly at low concentration levels. Thus, to overcome any overlapping of the 3-CA peak at low level and obtain good resolution, reducing the mobile phase to between 60 and 55% methanol and controlling the column oven at a temperature of 25 °C are recommended.

5.3.2 Detection limit of the studied compounds in the organic potato extract

In order to validate the extraction method, the LOD and LOQ for CIPC, IPC and 3-CA were calculated through repeated injection of 0.05 µg/mL of mixed standard solution prepared in organic potato extract. The results obtained from the HPLC analysis are presented in Table 5:3.

Table 5:3. LOD and LOQ values for replicate injections of a mixture of 0.05 µg/mL CIPC, IPC and 3-CA prepared by spiking organic potato extract.

Compound	LOD (µg/mL)	LOQ (µg/mL)
CIPC	0.003	0.010
IPC	0.019	0.064
3-CA	0.006	0.020

Data from this table can be compared with the data in Table 3:13 which gives the LOD and LOQ values for repeated injection of the same concentration (0.05 µg/mL) of a pure standard mixture. The values in Table 5:3 are close but slightly lower with the exception of IPC, which shows higher values due to the high variability of the small peak area resulting from the weak response at 210 nm. The limit of detection can also be affected by the presence of matrix interferences in the potato sample extract that can subsequently influence the quantitative measurement of the intended compounds.

The values in the Table 5:3 can be converted approximately to mg/kg of fresh potato tuber weight as explained in the equation below using the total solvent volume (40 mL) used to extract 5 g of peel, assuming that the peel represents approximately 10% of the total potato fresh weight (see Tables 5:5 and 5:6). In a recent study, the typical percentage peel weight was found to be 10 – 16% of whole potato tuber fresh weight (Oteef, 2008). Previous workers reported that the residue of 10 – 20 µg/mL of CIPC on a peeled potato basis is equivalent to 1 – 2 mg/kg on a whole tuber basis (Corsini et al., 1979; Brajesh and Ezekiel, 2010).

$$\text{LOD or LOQ value as mg/kg} = \frac{\text{value as } \mu\text{g/mL} * 40 * 10 * 1000}{5 * 100 * 1000}$$

The estimated values of LOQ as mg/kg based on fresh potato tuber weight (10%) are 0.01, 0.05 and 0.02 of CIPC, IPC and 3-CA respectively. Generally, these values are acceptable for the quantitative determination of CIPC and 3-CA residues at low levels in potato peel extract. No clean up step is required other than filtration, however, to obtain lower values for the LOQ a further clean up may be useful. A clean up step is important to avoid matrix interferences and obtain a lower limit of detection (Stajnbaher and Zupancic-Kralj, 2003).

5.3.3 Assessment of the recoveries of CIPC, IPC and 3-CA from spiking organic potato peel

Recovery information for the analyte following spiking of a sample is an important measurement during validation of an analytical method. In practice, variations in recovery are most apparent in the determination of pesticide residues in complex matrices such as foodstuffs and environmental samples (Thompson et al., 1999).

In order to evaluate the efficiency and the accuracy of the new method of methanol-soaking-HPLC for measurement of CIPC, IPC and 3-CA in potato sample, the recovery was investigated as a part of the method validation. This experiment was conducted by spiking organic potato peel with a solution of CIPC and 3-CA at three concentration levels 0.1, 1.0 and 10 µg/mL as described in Table 5:1. After extraction, the recovery was measured and compared with the recovery obtained from the control, which involved adding the spiking solution to empty bottles (no peel) as presented in Table 5:4.

Table 5:4. The recoveries of CIPC, IPC and 3-CA from spiking potato peel using the methanol-soaking-HPLC method.

Treatment	Conc. µg/mL	Compound	Recovery%	RSD% (n = 5)
Control	0.1	CIPC	100	5.7
		IPC	91	5.6
		3-CA	93	11.2
	1.0	CIPC	103	1.2
		IPC	99	0.5
		3-CA	100	2.3
	10	CIPC	99	2.7
		IPC	101	0.8
		3-CA	97	2.0
Spiking peel	0.1	CIPC	96	7.3
		IPC	90	9.8
		3-CA	ND	ND*
	1.0	CIPC	95	2.3
		IPC	89	2.3
		3-CA	10	20.8
	10	CIPC	89	3.3
		IPC	89	0.3
		3-CA	23	5.9

ND*: not detected (below the limit of detection)

Recovery values in this table represent the ratio of the amounts extracted and measured from the total amount added to spiked peel. The recoveries obtained from the control and spiked peels are more than 89% for both CIPC and IPC with good RSD% for five replicates at the three concentration levels (0.1, 1.0 and 10 µg/mL). In contrast and contrary to expectations, the recovery results of 3-CA from spiking potato peel was found to be 10 and 23% at concentration levels of 1.0 and 10 µg/mL respectively, with no peak detected for 3-CA at the lower level of 0.1 µg/mL. The control recoveries of 3-CA were greater than 93% at all three levels confirming that there was little or no adsorption onto the glass surfaces of the jar or possible losses through volatilisation (which will be discussed in details in Section 6.3.7).

Generally, there are several sources that can affect recovery measurements introducing systematic and random errors (Thompson et al., 1999). Typically, most analytical methods depend on extraction of the analyte from a complex matrix into a simple solution that is presented for the instrumental measurement. Incomplete extraction or strong binding of the analyte results in a value lower than the actual amount in the original sample. Additionally, in some procedures, presenting the extracted solution for measurement involves using a clean up step, filtration, C₁₈ column and concentrating the extract by rotary evaporation. These steps may also cause a loss of the analyte and subsequently give lower recoveries. Another possible explanation for the loss of the analyte may be due to volatilisation, solution transfer and adsorption onto laboratory glassware (Thompson et al., 1999; LeDoux, 2011).

In the present study, the recoveries for CIPC and IPC are acceptable. On the other hand, the reason for the low recovery of 3-CA is not clear and difficult to explain, but it might be attributed to incomplete extraction of 3-CA that is covalently bonded or strongly bound to the potato peel.

The recovery was tested at three concentration levels to assess how the recovery may depend on concentration. This was obviously the case on looking at the recovery of 3-CA, which showed decreasing recovery when the spiking concentration was decreased. In particular, at the lowest level of spiking there was no 3-CA was detected. Thompson et al., (1999) explained that the recovery might be close to zero at very low levels due to largely chemisorption of the analyte onto a limited number of sites on the sample matrix. Whilst, at high concentrations the recovery is partial, depending on the fraction adsorbed of the total analyte but at very high concentration this fraction is small and the recovery possibly will be efficiently complete and close to 100%.

One of the more significant findings to emerge from this recovery study is that spiking potato samples for 1 hour with a spiking solution of CIPC and 3-CA at a concentration level of 1 µg/mL which is equal to 8 µg/g in the potato peel (0.8 mg/kg in the whole potato tuber), the recovery after extraction will be acceptable for CIPC (95%), but it is only 10% for 3-CA. The low recovery of 3-CA from potatoes was investigated further (See Chapter 6).

5.3.4 Variability of CIPC residues and uniformity of a mixed peel sample

Routine analysis for the determination of CIPC residues in representative samples of potato tubers can be achieved by applying two procedures. Replicates of several potato tubers are taken, followed by analysis of each tuber individually then calculating the average results. Another alternative is pooling the replicate samples from these several potatoes and using a single analysis to get the average result. The second option is less time consuming and cheaper but the drawback is ensuring adequate mixing of the individual samples and the loss of information on residue variability between the individual potatoes. The variability information is of particular relevance to the maximum residue level (MRL) whilst simultaneously achieving adequate control of sprouting.

Since all the residue of CIPC is located in the potato peel, taking the potato peel to measure CIPC representing the whole potato tuber will be easier and is acceptable from an analytical point of view. The appearance of matrix interferences present in other layers of the tuber can be avoided as well (Oteef, 2008). When mixing peel from several potatoes the average result will be influenced by the size of individual potatoes as this affects the surface area to volume ratio and in addition the thickness of the potato peeling itself. The variability is averaged out by mixing the peel sample and therefore it is important to peel the whole potato for analysis.

The aim of this experiment was to assess the variability in CIPC residue levels resulting from mixing pooled samples of peel, taken from several potato tubers. The variability of the residue of CIPC was assessed using ten replicates of 5 g of peel taken from a pooled peel sample obtained from three potatoes treated with CIPC (see Table 5:5).

Table 5:5. Total fresh weights of three potato tubers, related total peel weights and the peel percentage.

Wt of tuber (g)	Wt of total peel (g)	Peel % in tuber
218.51	21.41	9.80
263.96	20.64	7.82
232.10	20.15	8.68
Mean		8.70

These three potatoes were approximately the same size so their peel percentages are close which practically depends on the peeling process and the peel thickness. Peeling a potato with a hand peeler and chopping the peel with a sharp kitchen knife into small and homogenous pieces are important to obtain good uniformity of the peel sample and to lower the variability in the analysis. Chopping the peel into fine pieces is important to increase the surface area and subsequently increase the efficiency of the extraction due to more contact between the solvent and the residue of CIPC in the peel. Prior studies have noted the importance of peeling by hand or using a mechanical peeler at 2 – 3 mm thick to obtain uniformity in the thickness of the peel, then chopping with a sharp kitchen knife into fine pieces (about 0.5 x 0.5 cm) and homogeneous mixing by hand to reduce the variability (Oteef, 2008; Corsini et al., 1979; Singh et al., 2011; Baloch, 1999).

The residues of CIPC in the ten replicates of the pooled peel sample were calculated as mg/kg in the whole tuber using 8.7% peel as the mean peel percentage for the three potato tubers presented in Table 5:5 (see Section 5.3.2). The variability of CIPC residue in the potato peel extract and whole tuber of these ten replicates is expressed by RSD% as shown in the Table 5:6.

Table 5:6. The RSD% values of CIPC residue in ten replicates of potato peel extract and whole tuber.

Sample no.	Wt of peel sample (g)	Solvent volume (mL)	Conc. of CIPC	
			Extract (µg/mL)	Tuber (mg/kg)
1	5	40	2.44	1.70
2	5	40	2.68	1.87
3	5	40	2.80	1.95
4	5	40	2.70	1.88
5	5	40	2.72	1.89
6	5	40	2.65	1.84
7	5	40	2.34	1.63
8	5	40	2.45	1.71
9	5	40	2.53	1.76
10	5	40	2.60	1.81
RSD%			5.65	5.65

As can be seen from the table the residue concentrations in the ten replicates are in close agreement (RSD% 5.65) showing the uniformity of the peeling, chopping, mixing and pooling of the peel samples from these three potato tubers.

It is recognised that there may be variability in CIPC levels within a single tuber according to the distribution on the potato surface. The eyes on the potato surface possess a high surface area resulting in higher uptake of CIPC (Singh et al., 2009). Therefore, it is expected that the residue level of CIPC will be much higher in the bud end of the tubers compared to the stem bud, owing to the bud end of the tuber containing more eyes than the stem bud.

Baloch (1999) investigated the variability of the distribution of CIPC residue within the potato tuber according to the location of the treated tuber in a box store. The residue level of CIPC was found to be higher in the upper parts compared to the lower parts of the tubers.

Oteef (2008) examined the variability in the residue of the sprout inhibitors 1,4-DMN in the peel surface of seven treated potato tubers by dividing each tuber into four quarters and analysing the peel of each quarter separately. There was some variability within the tuber expressed by %RSD in the four quarters of each tuber ranging from 5.03 % to 21.55 % whereas the variability between the tubers was 12.97 as RSD%. The author suggested for rapid analysis with minimum variability of the residue of 1,4-DMN in potato tubers that using several samples taken (as discs by a corer) of the peel from different locations in the tuber may well be an acceptable alternative to taking the peel from the whole tuber.

It should be noted that the deposition and the uptake of CIPC can be variable between varieties. This difference is due to the differences in the morphology of the periderm of these varieties which possess different types of surface (rough or smooth) and therefore surface area. A potato variety which has a rough surface and therefore a high surface area may end up with a high uptake of CIPC (Mondy et al., 1992b).

The ability to produce a uniform peel sample for a single tuber or several tubers is an important requirement in the method validation procedure (discussed in Section 5.3.5) and for routine residue analysis.

5.3.5 Final validation of the methanol soaking-HPLC method

To validate the new method of extraction of CIPC from potato samples, it was compared with the hexane-Soxhlet-GC method, which is the routine method used to extract and analyse CIPC residues in commercial potato samples. The comparison involved correlation between the CIPC residues extracted from 29 treated potatoes tubers by each method in addition to comparison between the residues of 3-CA.

5.3.5.1 Correlation between the developed method and the hexane Soxhlet–GC method for residue analysis of CIPC

Figure 5:2 shows representative chromatograms of the two analyses of the residue of CIPC from individually treated potato tubers.

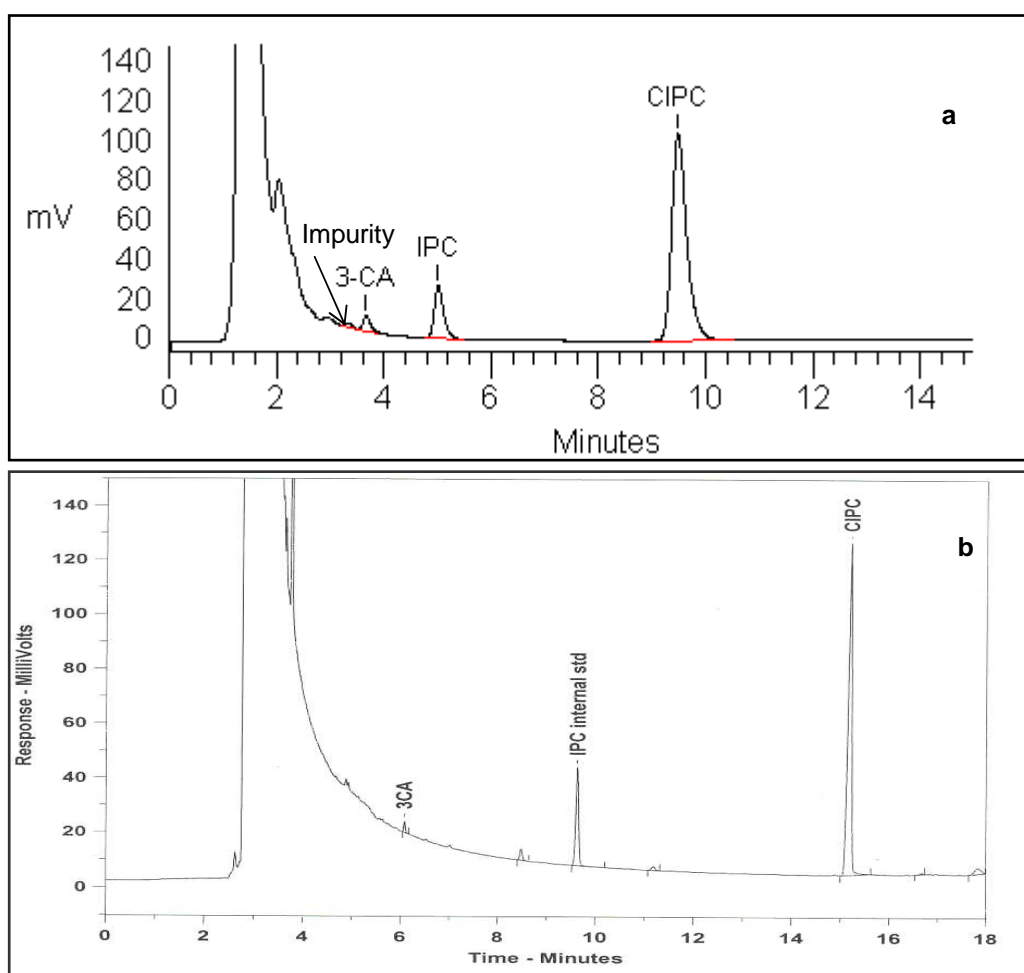


Figure 5:2. Typical chromatograms for analysis of CIPC from treated potato tubers applying: a- the methanol-soaking-HPLC method and b- the hexane-Soxhlet-GC method.

As shown in Figure 5:2, the chromatograms for the two analyses show good separation of CIPC and 3-CA in extracted treated potato tubers using IPC as the internal standard. It can be seen that only small interference peaks were present in the GC chromatogram, none of

which interfered with the identification and detection of the three compounds. However, the co-extractive peak from the potato peel caused a small effect on identification of the baseline of the small peak of 3-CA, which eluted earlier than the internal standard and CIPC (as expected, according to their polarity).

To assess the efficiency of the new method of methanol-soaking-HPLC, a comparison between the CIPC residues from the two analyses was made through the regression line as shown in the Figure 5:3.

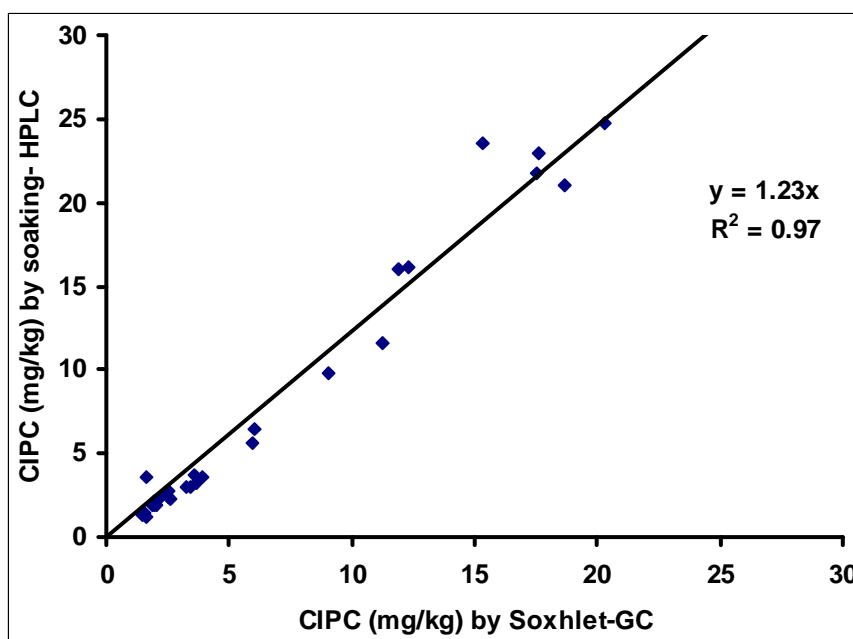


Figure 5:3. The correlation between CIPC residues in treated potato tubers as determined by methanol-soaking-HPLC and hexane-Soxhlet-GC.

As can be seen from the regression line, a good correlation was achieved as shown by the coefficient of the determination (R^2) of 0.97. The slope of the regression between the two analyses is 1.23 meaning that the new method of methanol-soaking-HPLC produced results for CIPC residue that were greater by 23% relative to the hexane-Soxhlet-GC method.

It is clear that there are several sources of difference between the two methods including the weight of the peel sample, extraction procedure, extraction time, extracting solvent, different standard solutions and different chromatographic analysis. Generally, these factors are summarised by the three main issues for developing any analytical method that are: extraction, clean up and analysis. The Soxhlet extraction procedure includes many steps and each of these steps has the potential to increase the analytical error (Wallis and Foley, 2005).

However, to investigate the role of the chromatographic analytical technique using the same extraction method, diluted extracts produced by the Soxhlet extraction were also analysed by HPLC to measure the residue of CIPC and compare with the results from the GC analysis. The comparison involved first preparing standards for each method to see if standards prepared in the different two solvents gave the same results. Standard solutions in each of methanol and hexane were prepared as five replicates of a 1 $\mu\text{g/mL}$ of mixture CIPC, IPC and 3-CA in order to examine the resolution, peak shape and peak area of compounds in hexane compared with methanol. Each solution was injected twice and the chromatograms obtained from HPLC analysis are shown in Figure 5:4.

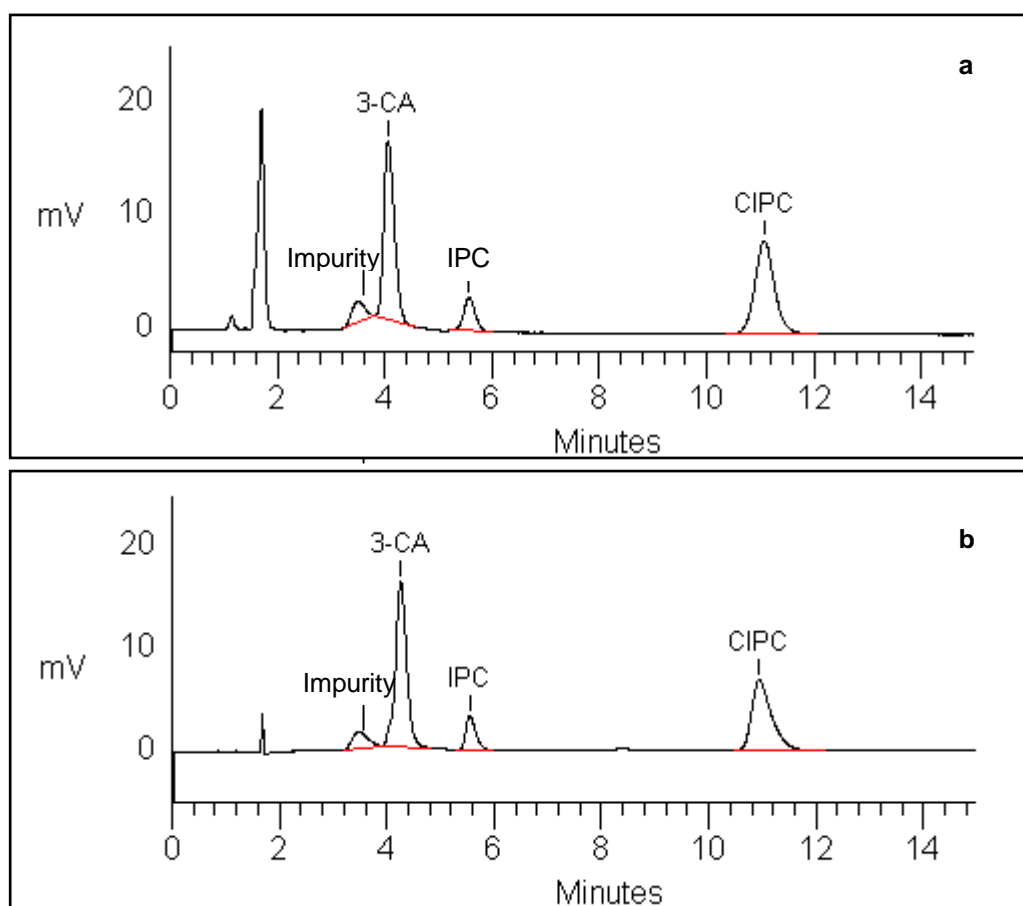


Figure 5:4. Chromatograms of the 1 $\mu\text{g/mL}$ standard solutions of CIPC, IPC and 3-CA prepared in: a-methanol and b- hexane.

On comparison of the two chromatograms, it can be seen that standards prepared in both methanol and hexane showed good resolution of the 3-CA, IPC and CIPC peaks which appeared at the same retention times (approximately 4.5, 5.5 and 11 respectively). However, the 3-CA peak showed a little overlapping with an impurity peak particularly for the methanol standard. In contrast, the hexane solution chromatogram showed a little

asymmetry and peak broadening for CIPC, in addition to gradual reduction of retention time especially after several injections (see Figure 5:5).

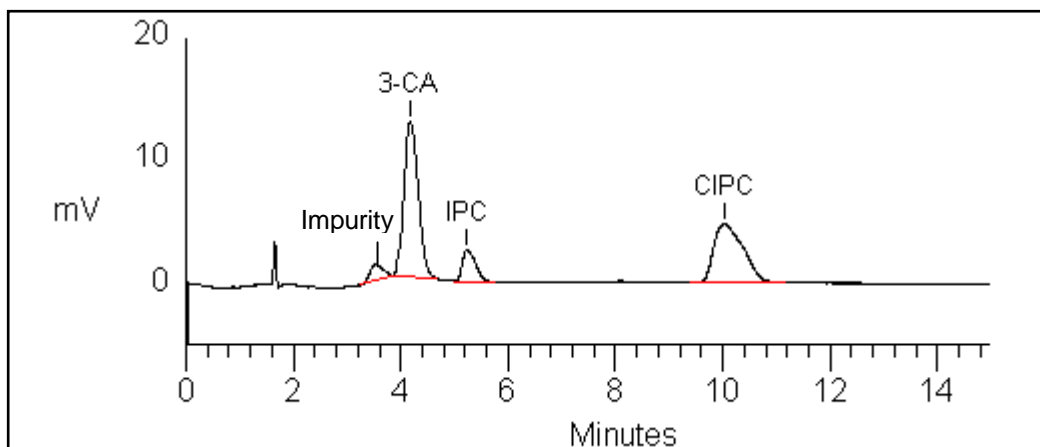


Figure 5:5. Chromatogram of 1 µg/mL standard solution of CIPC, IPC and 3-CA prepared in hexane after several injections.

The reason for this may be explained by the fact that methanol and hexane are immiscible solvents due to differences in polarity, viscosity and solubility. Technically, one of the common reasons for anomalous peak shape in HPLC analysis is injecting a sample or standard in a solvent that is different from that used for the mobile phase (Keunchkarian et al., 2006). For that reason, theoretically, hexane should not be injected into a non-miscible mobile phase such as methanol, but from a practical viewpoint, it depends on the injection volume of the sample and the mobile phase concentration. Experimentally, it is recommended starting with a very small volume then working up to a 20 µL injection. Some analysts have attempted to develop a method by injecting 5 µL of a toluene sample into methanol and buffer solution as a mobile phase and obtained good results for peak shape (John Dolan, personal communication). It was observed that in RP-LC injection a large volume of sample solvent non-miscible with the mobile phase could be used but resulted in both a gradual reduction of retention time and peak quality (Medvedovici et al., 2007).

In this study, the autosampler was set up for a 20 µL injection volume, which may have caused the peak shape deterioration shown. The strength of the mobile phase used was 62% methanol, using a lower concentration might improve peak shape, but a longer run time would be required.

A t-test was used to analyse the relationship between the standards prepared in the two solvents through analysis of the peak area of the standards. As can be seen from Table 5:7,

the t-test results did not show any significant difference ($p > 0.05$) between standard preparations for IPC in the two solvents. However, there was a highly significant difference ($p < 0.001$) between the two preparations for CIPC and 3-CA.

Table 5:7. The mean of peak area and t-test result for each compound prepared in solutions of 1 µg/mL of methanol and hexane.

Compound	Mean peak area (n = 10)		t-test
	Methanol	Hexane	
CIPC	19921670	18431946	HS*
IPC	4102315	4115729	NS*
3-CA	20853940	22576297	HS

HS*: high significant difference ($p < 0.001$), NS*: no significant difference ($p > 0.05$)

There are several reasons for these differences; the main reason is overlapping of the impurity with the 3-CA peak in the methanol solution which affected the detection of exact peak areas. Another possible explanation for this difference is related to the immiscibility of hexane in the methanol mobile phase as discussed above. Although, the peak areas of CIPC and 3-CA in both preparations show a statistically significant difference, practically this small random variability could be due to a weighing error during the preparation; in particular, the preparation of stock solutions (10 000 µg/mL) of compounds in methanol and hexane were not made at the same time, which can result in a larger variability in weight error and preparation conditions. Therefore, these reasons clearly support the confirmation of using these standard solutions for HPLC analysis provided the standards are prepared in the same solvent as the samples.

The dilute extracts from the Soxhlet extraction (before concentration by rotary evaporation) were analysed by HPLC to determine the CIPC residue. Figure 5:6 shows a typical chromatogram obtained from HPLC analysis for the Soxhlet extracts.

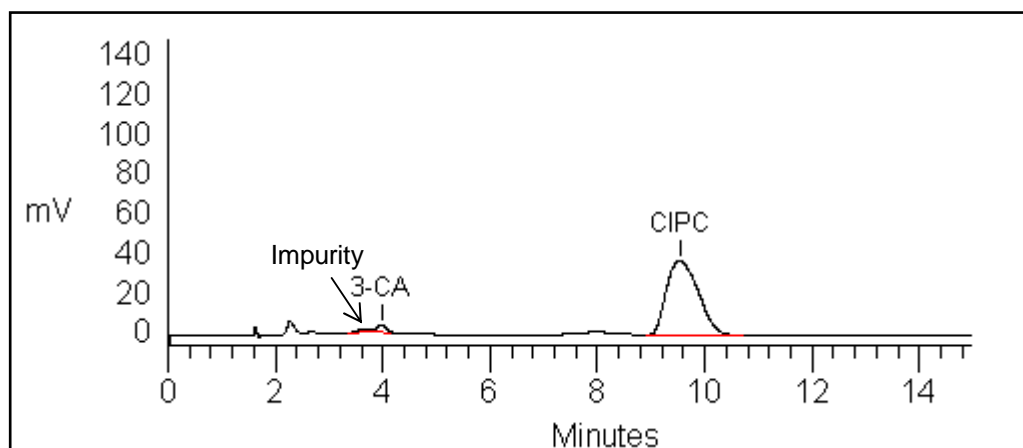


Figure 5:6. Typical chromatogram for HPLC analysis of the Soxhlet extract of CIPC residue from treated potatoes.

Although the impurity peak slightly overlapped with the 3-CA peak, the two peaks of 3-CA and CIPC were well separated with good resolution but reduction in their retention times. Even though the CIPC peak was broadened and had a low peak height (as discussed above), this did not effect the CIPC residue measurement. The analysis of the hexane-Soxhlet extract by HPLC did not involve using the internal standard as apparent in the chromatogram in Figure 5:6. This is likely because the extract was made up to volume (100 mL) with hexane prior to analysis by HPLC.

A comparison was also made between the HPLC and GC analyses of the CIPC residues extracted by Soxhlet extraction through linear regression as shown in Figure 5:7.

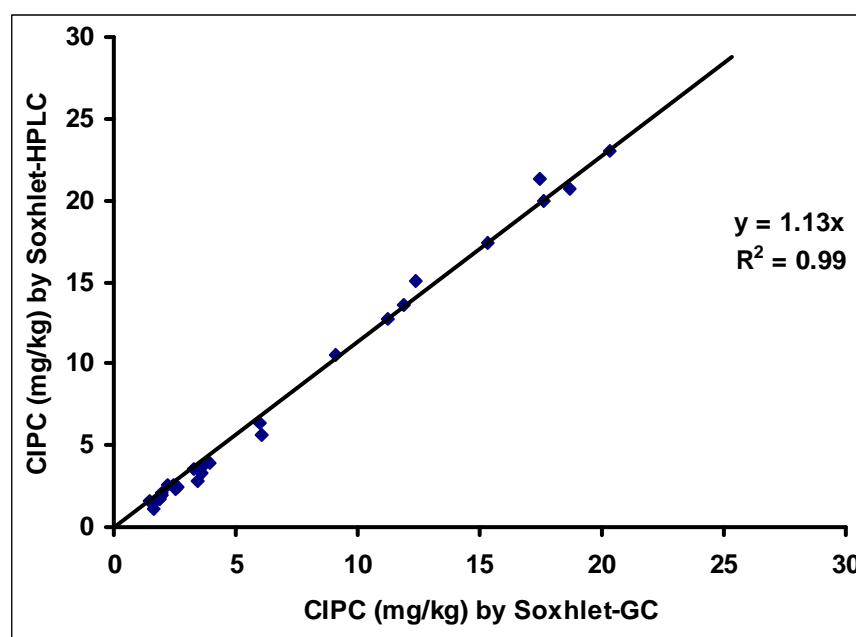


Figure 5:7. The correlation between CIPC residues in treated potato tuber (by Soxhlet extraction) as determined by both HPLC and GC analysis.

It is apparent from the figure that the correlation between the two analyses shows good agreement through the coefficient of determination of R^2 (0.99). However, despite the same extract of Soxhlet extraction and the same stock standard solution being used for both analyses, the recovery between the two analyses of the extract is different, showing 13% lower results for GC analysis.

The main difference between the two procedures is that rotary evaporation used to concentrate the extract for the GC analysis. It is possible that the loss is due to the transfer of the extract from the round bottom flask to the 2 mL volumetric flask, insufficient rinsing of the extract flask with solvent may leave some residue of CIPC on the surface. Another possible explanation for this loss is volatilisation of CIPC during solvent evaporation using the rotary evaporator. It was presumed that one of the principal sources for loss of the residue of the potato sprout inhibitor 1,4-DMN from potato extracts by homogenisation extraction followed by GC analysis might be during the solvent evaporation stage (O'Hagan, 1991; Beveridge, 1979). Therefore, it was recommended to control the temperature of the water bath and the pressure using constant vacuum in the rotary evaporator during evaporation of the hexane solvent to prevent the loss of 1,4-DMN.

In order to check the loss of CIPC in this study, it is suggested that re-extracting the round bottom flask as well as rotary evaporation of a standard solution could be carried out. The role of rotary evaporation in the loss of 1,4-DMN from the Soxhlet extract was investigated through a recovery experiment by reducing 100 mL of a standard solution of 1,4-DMN in hexane to about 2 – 3 mL using rotary evaporation at a temperature of 35 °C (Oteef, 2008). Analysing the concentrate of a standard solution by HPLC showed a loss of 1,4-DMN of 9% during rotary evaporation. The author interpreted this loss as due to the temperature of the water bath used during the rotary evaporation.

To assess the contribution of the extraction method to the higher residue results from the new method of methanol-soaking-HPLC, the correlation between the two HPLC analyses of methanol-soaking and hexane-Soxhlet extract was tested as illustrated in Figure 5:8.

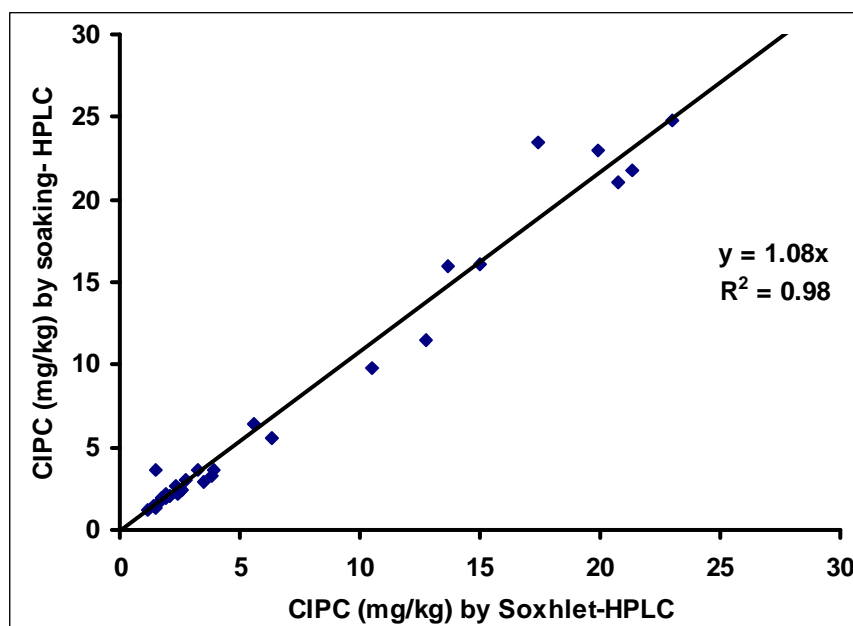


Figure 5:8. The correlation between CIPC residues in treated potato tuber determined form methanol-soaking-HPLC and hexane-Soxhlet-HPLC analyses.

The figure shows a good fitting regression line of CIPC residue obtained by the two extraction methods expressed by the coefficient of determination R^2 (0.98). However, the methanol-soaking extraction gave results approximately 10% greater than hexane-Soxhlet extraction. A possible explanation for this might be related to the solvents used in each extraction, methanol has a higher polarity compared with the non-polar solvent hexane. Methanol as a polar solvent is more efficient than hexane in extracting the organic compounds from plant materials in particular polar compounds. That may be explained by the axiom of “like dissolves like”, as CIPC is a polar compound; its solubility and dipole-dipole interaction in polar extractants are much higher than in less polar extractants (Sun and Lee, 2002). Another possible reason is that the contact time between the CIPC residue in the peel and methanol using soaking extraction was greater (~ 16 hours) than with hexane in Soxhlet extraction.

To investigate these assumptions, examining the use of methanol with Soxhlet extraction and in contrast hexane with soaking could be undertaken to examine further the difference between the two extractants.

This comparison indicates that the higher efficiency of the new method of methanol-soaking-HPLC compared to the standard method of hexane-Soxhlet-GC is owing to both chromatographic analytical technique and extraction method.

Following the analysis, the results of residue levels in the 29 individual potatoes obtained from the three analyses are presented in Table 5:8.

Table 5:8. The range of CIPC residues in 29 treated potatoes measured by three analytical methods.

Analysis method	Range of the residue (mg/kg)	Mean \pm SD (n = 29)
Methanol-soaking-HPLC	1.16 – 24.79	7.70 \pm 8.08
Hexane-Soxhlet-HPLC	1.13 – 22.97	7.19 \pm 7.27
Hexane-Soxhlet-GC	1.48 – 20.34	6.55 \pm 6.16

It should be pointed out that the residues of CIPC in this table are not representative of typical residues found in potatoes from potato stores. These samples were selected to provide a good range of CIPC residues up to approximately double the MRL value (10 mg/kg) for the purpose of validating the new analytical method.

As a conclusion to the present work, a comparison between two analytical methods showed that the proposed method of methanol-soaking-HPLC has the following advantages over the hexane-Soxhlet-GC: rapid, with straightforward sample preparation, easy analysis, less involved laboratory procedure, less solvent consumption, lower cost, greater sensitivity, a satisfactory run time and no requirement for rotary evaporation and Soxhlet apparatus. Moreover, the new method saves on water required for the cooling systems of both the Soxhlet apparatus and the rotary evaporator. Therefore, the methanol-soaking-HPLC method confirms its superiority over the traditional hexane-Soxhlet-GC method. The new developed method is suitable to apply to the routine analysis of potatoes treated with CIPC and allows the analysis of 20 potato samples per day. Practically, a short analysis time is desired for environmental samples due to the huge number of samples to be analysed every day.

5.3.5.2 Summary of methanol-soaking-HPLC method

Procedure

The final method developed in this chapter for the determination of CIPC residues in potato samples is summarised below:

1. Potato tubers are washed and dried.
2. The weight of each tuber is recorded.
3. After peeling the potato with a stainless steel peeler, the weight of peel is taken.
4. The peel is chopped into fine pieces and mixed to obtain a homogenous sample.
5. 5 g of chopped peel sample from the potato tuber is weighed into a 100 mL screw top jar, then 40 mL of methanol containing the internal standard of 10 µg/mL of propham (IPC) is added as the extracting solution.
6. The samples are left soaking overnight (~ 16 hour) at room temperature.
7. The extract is filtered and transferred into an HPLC vial through a 0.2 µm PTFE membrane syringe filter and analysed.

Chromatographic conditions

The chromatographic parameters for this method are summarised as follows:

- Column: Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 µm Sphercclone)
- Guard column: Phenomenex[®] Security GuardTM
- Detector: SpectraSERIES UV100
- Wavelength detection: 210 nm
- Mobile phase: 62 % methanol
- Flow rate: 1.5 mL/min

- Chromatographic run: 15 minutes.
- CIPC retention time: ~12 minutes.
- IPC retention time: ~ 6 minutes.
- 3-CA retention time: ~ 5 minutes
- Injection volume: 20 μ L
- Column temperature: 25 ° C

Calculation of the residue of CIPC

$$\text{Conc. in tuber (mg/kg)} = \frac{[\text{Conc. in extract} * \text{Vol. of extract} * \text{Wt of total peel}]}{[\text{Wt of peel sample} * \text{Wt of potato tuber}]}$$

5.3.5.3 Determination of 3-CA in commercial potatoes samples treated with CIPC

The new method was also tested for the extraction of the CIPC breakdown product 3-CA from the same potato tubers used in the experiment in Section 5.3.5.1.

The results obtained for the residue of 3-CA from the new method of methanol-soaking-HPLC were compared with hexane-Soxhlet-GC analyses as shown in Table 5:9.

Table 5:9. Residues of 3-CA in 29 potatoes tubers treated with CIPC and determined by the two methods of methanol-soaking-HPLC and hexane-Soxhlet-GC.

No. of tuber	3-CA (mg/kg)	
	Methanol-soaking-HPLC	Hexane-Soxhlet-GC
1	0.06	0.05
2	0.10	0.09
3	0.11	0.08
4	0.18	0.12
5	0.18	0.08
6	0.07	0.11
7	0.06	0.12
8	0.10	0.12
9	0.08	0.08
10	ND*	0.14
11	0.11	0.22
12	ND	0.11
13	0.30	0.07
14	0.15	ND
15	0.33	0.12
16	0.18	0.06
17	0.34	0.08
18	0.06	ND
19	0.12	ND
20	0.10	ND
21	0.06	ND
22	ND	ND
23	ND	ND
24	ND	0.05
25	ND	0.07
26	0.07	0.08
27	0.06	0.04
28	0.13	ND
29	ND	ND
Mean \pm SD	0.10 \pm 0.09	0.07 \pm 0.06

ND*: not peak detected

It is apparent from the table that very low concentrations of 3-CA were detected by the two different analyses, with high variability between the tubers. Additionally, some tubers showed no detection of 3-CA residue since no peak appeared in the area of the retention time of 3-CA in both analyses. The residues ranged from 0.06 to 0.34 mg/kg for the methanol-soaking-HPLC analysis whereas between 0.04 and 0.22 mg/kg was detected by the hexane-Soxhlet-GC method. The lowest concentrations reported are higher than the limit of quantification (LOQ) for the methanol-soaking-HPLC method (0.02 mg/kg as described in Section 5.3.2). In contrast, the LOQ value for the hexane-Soxhlet-GC method is not available since this method is validated only for extraction and analysis of CIPC and not for 3-CA.

Figure 5:9 shows the regression plot to evaluate the correlation between the residues of 3-CA by the two analytical methods.

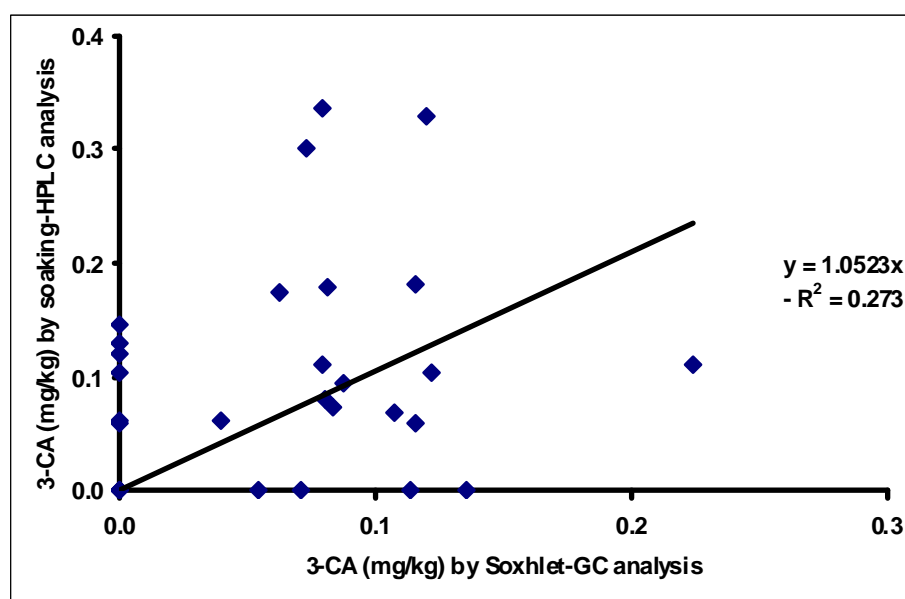


Figure 5:9. Shows the correlation between the residue values of 3CA from potato samples treated with CIPC and analysed by two methods of methanol-soaking-HPLC and hexane-Soxhlet-GC.

This figure shows very poor correlation of the residues of 3-CA between the two methods for each individual potato sample. That contrasts with the evaluation of the residue of CIPC which showed a good correlation between the two methods on analysis of the same potato tubers.

An interesting finding in this study was that despite the low recovery for 3-CA, it was actually identified in the potato peel extracts as a small peak in the HPLC chromatogram. It

was assumed that this small residue level of 3-CA in potatoes was of no concern, but the unanticipated low recovery found in this study is a noteworthy issue indicating that the residue may be much higher. As the concentration found for 3-CA represents less than 10% (recovery% at 1 µg/mL concentration level) of the actual amount present in the potato sample, a residue of 0.3 mg/kg (e.g. as shown in Table 5:9) could represent a concentration of 3 mg/kg or more in the tuber.

5.4 Conclusion

A robust method based on a methanol-soaking overnight extraction (16 hours) coupled with HPLC-UV was developed for the extraction and determination of the potato sprout inhibitor CIPC in potatoes using IPC as the internal standard.

The limit of quantification was estimated to be 0.01, 0.05 and 0.02 mg/kg in whole tuber for CIPC, IPC and 3-CA respectively. The efficiency of the new method was assessed through a recovery study of spiking organic potato peel at three concentration levels 0.8, 8.0 and 80 µg/g. The results demonstrated greater than 89% recoveries for both CIPC and IPC whereas the recovery results for 3-CA were between 10 and 23% at concentration levels of 8.0 and 80 µg/g respectively. No 3-CA was detected at the lowest concentration studied (0.8 µg/g). Therefore, the new analytical method described thus far is only suitable for CIPC and it is not fitting methodology for 3-CA.

The new method was validated through comparison with a standard hexane-Soxhlet-GC method. The proposed method showed results for CIPC residues that were approximately 23% higher than the hexane-Soxhlet-GC method. Partially, this increase was attributed to using a rotary evaporator to concentrate the extract (Soxhlet-GC method), where volatilisation may result in the loss of CIPC. The polarity of the solvents used is also considered a possible explanation for the discrepancy between the two methods.

The new method is easy to use, efficient, inexpensive, rapid and appropriate to determine the residue of CIPC in 20 potato samples per day. However, this study has shown that potentially high levels of 3-CA residues are present in commercial potatoes even though the method described has a very low recovery for this particular compound.

Therefore, considerable attention should be given to the development of a method for analysis for 3-CA. The work in the next chapter will focus on finding a suitable method for the extraction of this aromatic amine from potato tubers.

Chapter 6: Extraction method for the determination of 3-chloroaniline in potato samples

6.1 Introduction

Currently, there is a big consideration of the maximum residue level of CIPC which should include its degradation product 3-CA in potatoes hence there is a requirement to find a suitable extraction method for both chemicals. In the previous chapter, a simple method with excellent extractability for CIPC from potato samples was developed using methanol as the extractant, however, this method proved to have a low recovery for 3-CA. This poor extraction could affect the actual measurements for this compound in potato especially in peel samples. This unexpected result of low recovery has thrown up many questions regarding how 3-CA is held onto the potato peel and subsequently how to find a suitable means to improve its extractability. Therefore, further investigation is essential to answer these questions.

3-CA is an aromatic amine, the quantitative determination of this group of compounds from different environmental matrices generally shows an analytical challenge associating low extraction recoveries and difficult separation chromatography due to the physicochemical properties of volatility, polarity, basicity and water solubility being high (Oostdyk et al., 1993).

Several investigations have been reported on the fate of chloroaniline compounds in plants (Still et al., 1981; Balba et al., 1977; Kaufman et al., 1976; Kaufman, 1976). Most of these studies have encountered the problem of bound or unextractable residues of these compounds. "Bound residues are compounds in soil, plants, or animals which persist in the matrix in the form of the parent substance or its metabolites after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix" (Fuhr et al., 1998; Barriuso et al., 2008). Although, these studies have not attempted to counter the problem of bound residues and explain their nature or identity the role of biological and environmental effects on this binding, a few studies have pointed out that chloroanilines were bound to plants through lignin which is a major binding site for unextractable residues of these compounds (Lange et al., 1998; Still et al., 1981; Trenck et al., 1981; Yih et al., 1968).

Still et al., (1981) indicated that 3-CA and 3,4-dichloroaniline may translocate in rice plants and become covalently bonded to lignin when the rice plant was treated with these compounds. It was reported that more than 40% of the residue of these compounds remained in plant lignin. These results suggested that the high reactivity of the free aromatic amino group of 3-CA has a considerable role in the incorporation into lignin. Although the lack of the definition of a lignin structure is due to the variation in the structure of monomers the authors speculated that chloroaniline may bind with the carbon atoms in the monomer side chain or aromatic ring structure through a covalent bonding, or it may be trapped inside the cage of the lignin structure without chemical bonding.

Weber et al. (2001) reviewed that the rapid sorption of the aromatic amine on the sample surface (soil and sediment) is reversible and can be attributed to electrostatic interaction, hydrophobic partitioning and the formation of a Schiff base (e.g., imines), while the slower sorption is attributed to irreversible covalent binding.

Adrian et al. (1989) reviewed that in most cases a portion of the applied compounds in plant and soil cannot be removed by exhaustive solvent extraction. They showed that the substituted anilines in plant and in soil formed up to 95% of the bound residues under various conditions. The formation of these bound residues was not clear due to the complex structure of the biological matrix. Adrian et al. (1989) indicated that severe extraction methods in some cases are unsuitable because they can destroy the structure of the samples and the identity of the nonextractable residues. However, some gentle extraction methods can be employed with some success such as high temperature distillation, supercritical fluid extraction and pyrolysis.

Additionally, 3-chloroaniline is subject to microbial degradation by bacterial cultures supplied with suitable additional carbon sources, with atmospheric oxygen being required for the enzymatic reaction that initiates this degradative process (Janke et al., 1984; Ferschl et al., 1991). However, it was presumed that no microbial degradation of chloroanilines will occur during a short incubation period (Sihtmaee et al., 2010).

The pK_a of 3-chloroaniline is 3.52, indicating that this basic compound will primarily be present as the non ionic species in the environment (SRC, 2011). Therefore, the possibility of cation exchange or electrostatic interaction of the protonated organic amine (NH_3^+) with ions on the potato peel is not expected. Nevertheless, ion exchange of 3-CA will be

investigated in this study in terms of pH effect of the extracting solution on the recovery improvement.

Extraction of 3-CA from potato peel is an important issue which should be addressed, therefore, the main aim of the work in this chapter, as a first step, concentrates on improving the extraction recovery of 3-CA from potato sample through:

- Investigation of the effect of several factors on the extraction of 3-CA from potato peel including potato variety, extracting solvent, extraction method, spiking procedure, treatment of the potato sample before spiking and spiking of different parts of the potato tuber.
- Suggestion of possible hypotheses for the mechanism of binding of 3-CA to the potato peel.
- Investigation of the suggested mechanisms with the aim of improving the extractability of 3-CA.
- Optimising the extraction process through temperature and time factors.
- Assessing the suitability of a new extraction method on real potato samples treated with CIPC.
- Studying the effect of fogging temperature and the number of CIPC applications on the residue levels of CIPC and 3-CA in potatoes treated in potato stores.

6.2 Materials and Methods

6.2.1 Methods

6.2.1.1 Chemicals

3-Chloroaniline, propham, chlorpropham, chlorogenic acid, tyrosine, L-aspartic acid, ascorbic acid, ammonium hydroxide solution, dichloromethane, sodium sulphate anhydrous and lithium acetate were purchased from Sigma-Aldrich Chemi GmbH (Germany). Hexane, formic acid, malonic acid, L-asparagine, glutamic acid, arginine and sodium dithionite were purchased from BDH (UK). Glucose was obtained from Fluka

Chemical Company (Switzerland). Acetonitrile (HPLC grade), methanol (HPLC grade), chloroform, citric acid, sulphuric acid, sodium carbonate, sodium hydroxide, sodium chloride and ammonium acetate were supplied by Fisher Scientific International Company (UK). Caffeic acid was obtained from Lancaster (UK). Acetic acid was obtained from VWR international (France). See Section 2.1.1 for preparation of the standard solutions of CIPC, IPC and 3-CA in methanol, ACN and hexane.

6.2.1.2 HPLC analysis

The HPLC system used is described in Section 2.1.2 and the chromatographic conditions for the HPLC analysis method are summarised in Section 3.4.3.4 with the exception being that the mobile phase was reduced to 55% methanol where the analysis did not involve CIPC. The run time was 10 minutes and the retention times of 3-CA and IPC were approximately 6 and 8 minutes respectively. When using acid extractants, the run time was increased to 15 minutes keeping the same concentration (55% methanol) for the mobile phase.

6.2.1.3 Methanol soaking extraction

The same procedure for the soaking extraction method in Section 5.2.1.4 was applied. The weight of the peel sample and volumes of spiking and the extracting solutions in this chapter were reduced to half keeping the same spiking level (1 µg/mL) using 2.5 g of peel sample, 100 µL of 200 µg/mL of 3-CA spiking solution and a 1 hour spiking time. The volume of methanol extractant was reduced to 20 mL. The extract was filtered and transferred into HPLC vials through a 0.2 µm PTFE (Teflon) membrane syringe filter and analysed. The standard solution was a mixed solution of 1 µg/mL of 3-CA and IPC prepared in methanol and injected in duplicate. The extraction procedure also involved replicates of nonspiked peel as blank controls.

6.2.1.4 Soxhlet extraction

The Soxhlet extraction procedure described in section 5.2.1.5 was applied. A 12.5 g sample of chopped peel was placed into a Soxhlet thimble containing 10 g of anhydrous sodium sulphate and spiked with 500 µL of 200 µg/mL solution of 3-CA for 1 hour prior to extraction with 150 mL of extracting solvent for 3 hours. The extract was transferred into a 100 mL volumetric flask and made up to volume with extracting solvent before analysis by HPLC.

6.2.1.5 Calculation of concentration and recovery

The concentration of 3-CA and CIPC was calculated as described in Sections 5.2.1.4 and 5.2.1.5 taking into account the changes in the weight of the peel sample (2.5 g) and volume of extracting solvent (20 mL) in the case of the soaking extraction method.

The recovery of 3-CA was calculated according to either:

$$\text{3-CA recovery\%} = \frac{[\text{Conc. in extract } (\mu\text{g/mL})]}{[\text{Conc. in Std } (\mu\text{g/mL})]} * 100$$

Or (in the absence of internal standard in the extractant)

$$\text{3-CA recovery\%} = \frac{[\text{PA in sample}]}{[\text{PA in Std}]} * 100$$

Note:

PA: peak area

Std: standard solution

Conc.: concentration

6.2.2 Influence of potato variety on the extraction 3-chloroaniline

This experiment was conducted to examine the extraction recovery of 3-CA from different organic potatoes varieties (Maris Peer, Valor, Orla and Sante) purchased from a local supermarket. Five replicates of chopped peel of each variety were spiked with a solution of 3-CA prepared in methanol and left 1 hour. The spiked peel was extracted by the soaking method with methanol containing the internal standard of IPC (see Section 6.2.1.3).

6.2.3 Influence of solvent on the extraction 3-chloroaniline

The recovery of 3-CA was examined using different extracting solvents (acetonitrile, methanol, formic acid, dichloromethane and hexane). Potato peel was spiked with different spiking solutions of 3-CA prepared in different solvents (acetonitrile, methanol, water, hexane and hexane with Na₂SO₄). The extraction methods involved soaking and Soxhlet (see Sections 6.2.1.3 and 6.2.1.4). With Soxhlet extraction, the extraction time was 3 hours with the exception of formic acid where a 7 hour extraction time was applied due to its

high boiling point (100 – 101 °C) and therefore needs more time to reflux the solvent within the Soxhlet apparatus. The standard solution was prepared in the same spiking and extracting solutions but in some cases just in methanol (during using formic acid, dichloromethane and Na₂SO₄ hexane to avoid any deterioration of the column). The experiments were undertaken using five replicates for spiked peel in addition to another five replicates without spike to be used as the blank control.

6.2.4 Influence of extraction method on 3-chloroaniline recovery

Different extraction methods were applied to extract 3-CA from spiked peel samples (n = 5) using methanol as the extractant. With the exception of the Soxhlet extraction, the same ratio of chopped peel sample, spiking solution and extracting solution were used for all extraction methods. These extraction methods included:

Soaking: overnight soaking extraction (~ 16 hours) at ambient temperature.

Sonication: extraction for 30 minutes in a sonication bath at 50 °C.

Heating: extraction for 45 minutes in a water bath at 50 °C.

Stirring: stirring thoroughly on a magnetic stirrer for 1 hour.

Soxhlet: extraction for 3 hours in Soxhlet apparatus.

6.2.5 Influence of spiking time on the extraction of 3-chloroaniline from potato peel

In order to ascertain if the spiking time had any impact on the recovery measurements, a simple experiment was performed. The experiment involved spiking chopped peel (n = 5) with a methanol solution of 3-CA, then allowing it to stand for different lengths of time (0, 2 – 3, 5 and 60 minutes) prior to the overnight soaking extraction.

6.2.6 Influence of spiking solvent on the extraction 3-CA

This experiment was conducted to examine the spiking solvent used on extraction of 3-CA. Different solvents (water, methanol, chloroform and dichloromethane) were used to prepare spiking solutions of 3-CA at the same concentration level of 200 µg/mL. These solutions were used to spike chopped peel applying the overnight soaking extraction

process as described in Section 6.2.1.3. Five replicates of each of spiked and nonspiked peel were prepared for this experiment.

6.2.7 Extraction of 3-chloroaniline from spiked potato samples

The recovery of 3-CA from spiking treated potato samples and from different parts of the tuber was investigated. A series of experiments were conducted involving spiking the potato samples with a methanol solution of 3-CA following by overnight soaking extraction using a solution of IPC in methanol (see Section 6.2.1.3). Each experiment included five replicates of each of spiked and nonspiked samples. The potato samples tested were:

Fresh peel: fresh chopped peel.

Dried peel: 1 g of chopped peel dried in an oven at 100 °C overnight.

Dried peel rewetted: 1 g of the dried chopped peel was rewetted with 2 mL of deionised water and mixed for 2 minutes.

Peel treated with chloroform: Small and uniform sized potato tubers were treated by dipping them into a 500 mL beaker containing chloroform at room temperature for five minutes and immediately thereafter swirled in a second beaker of chloroform for another five minutes, allowing for the removal of all the wax from the skin. The potato tubers were placed in a fume hood overnight to evaporate the chloroform. The next day, 2.5 g of the chopped peel was spiked.

Peel treated with methanol: wet chopped peel was treated with methanol through Soxhlet extraction for 3 hours.

Brown surface side of peel: pieces of peel were spiked directly onto the outer (brown) surface of the peel.

White surface of peel: pieces of peel were spiked directly onto the white surface side of the peel.

Skin: the outer layer of the tuber obtained by peeling very thinly (see Figure 6:1).

Cortex: the layer between the skin and the vascular ring (see Figure 6:1).

Pith: the translucent part in the centre of the potato (see Figure 6:1).

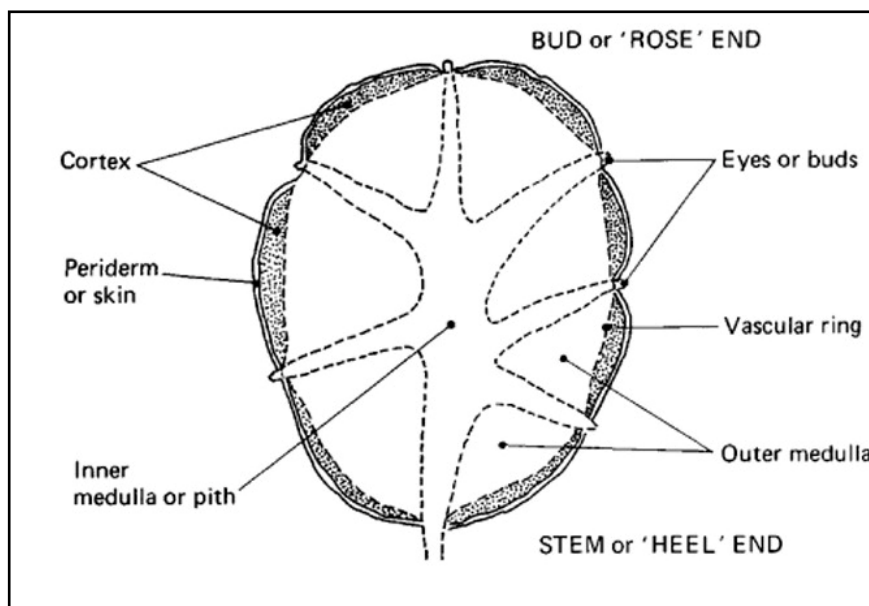


Figure 6:1. Cross section of the internal structure of a potato tuber (Woolfe, 1987).

6.2.8 Investigation of 3-CA volatilisation losses during spiking

6.2.8.1 Using empty jars without peel

An experiment was performed by measuring the recovery of 3-CA at two spiking levels of $2\text{ }\mu\text{g}$ ($100\text{ }\mu\text{L}$ of $20\text{ }\mu\text{g/mL}$) and $20\text{ }\mu\text{g}$ ($100\text{ }\mu\text{L}$ of $200\text{ }\mu\text{g/mL}$) using the same spiking and soaking extraction procedures and two solvents (methanol and acetonitrile) without potato peel. A spiking solution of 3-CA was added to an empty screw cap jar with lids ($n = 5$), after 1 hour, 20 mL of extracting solution was added to the spiking solution and left overnight.

6.2.8.2 Use of Tenax traps in collection system

Another experiment was carried out to investigate the possibility of volatilisation of 3-CA from the spiked peel using Tenax traps. The Tenax trap sample used in this experiment was a 105 mm long borosilicate glass tube with a 3 mm internal diameter and packed with Tenax GC resin as described in Section 2.1.10. Two Tenax traps were connected through two silicone rubber tubes to the top and bottom of a glass flask (5 L) as shown in Figure 6:2. The first Tenax trap was connected to the inlet tube in the bottom of the flask and to the air cylinder (BOC Glasgow Ltd.) to ensure there was no 3-CA in the air supply. The second Tenax trap was used to collect any 3-CA vapour released. This trap was connected to the outlet in the top of the flask which contained 25 g of chopped peel spiked with 1 mL of $100\text{ }\mu\text{g/mL}$ methanol solution of 3-CA. The flask was composed of two parts which

were fixed using three spring clamps and Teflon rings to prevent any air leaking from the collection system. The system was left for 24 hours in the incubator at a temperature of 20 °C and an airflow rate of 20 mL/min (controlled by Soap Bubble Flowmeters). These extreme conditions were used to allow complete evaporation of 3-CA at a constant temperature of 20 °C and to keep the system closed thus avoiding any contamination effect. The Tenax traps were then eluted with 10 mL of acetonitrile and the solution analysed by HPLC.

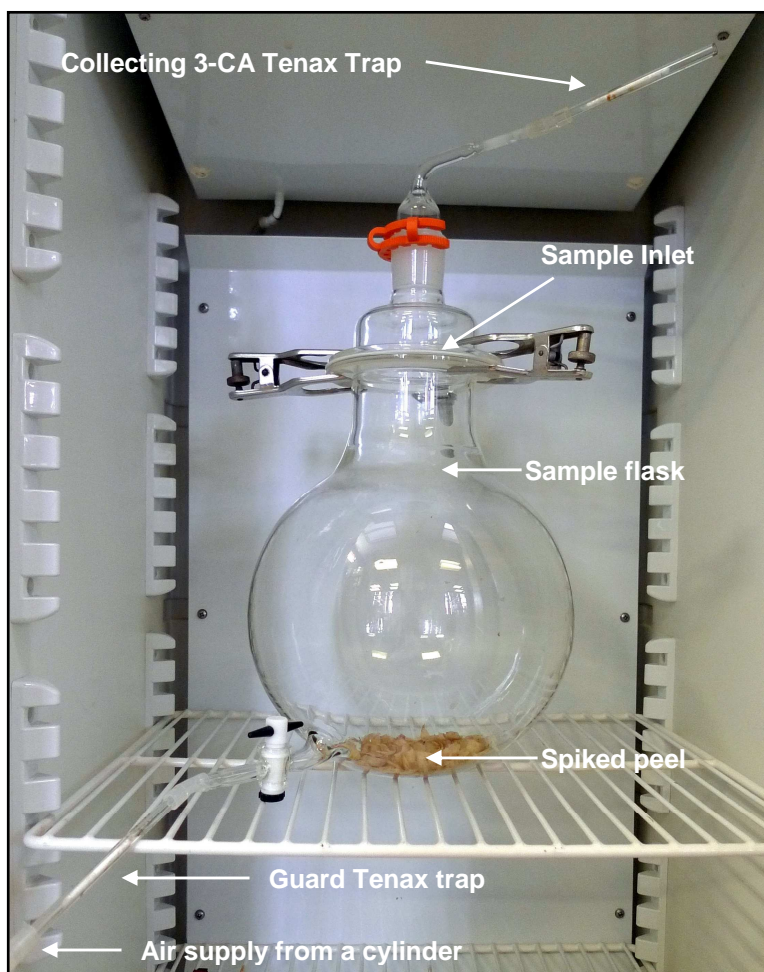


Figure 6:2. The collection system for 3-CA from spiked potato peel.

6.2.9 Investigation of the loss of 3-CA by reaction with different potato chemical components

6.2.9.1 Reaction with glucose

This trial was conducted spiking different weights of glucose (mixed with 1 mL water) with 100 µL of a 200 µg/mL solution of 3-CA for 1 hour prior to overnight soaking and extracting with 20 mL of methanol solution containing 1 µg/mL IPC. The experiment also included spiking 1 g from glucose in absence of water.

6.2.9.2 Reaction with other potato chemical components

This part of the work involved a series of experiments examining the recovery of 3-CA by spiking with different chemicals present in potato composition namely: chlorogenic acid, caffeic acid, asparagine, citric acid, aspartic acid, glutamic acid, arginine, tyrosine, malonic acid and ascorbic acid. Aqueous solutions of 100 µg/mL of each compound were prepared. 200 µL of each solution was mixed separately with 200 µL of 100 µg/mL of aqueous 3-CA solution and left for 1 hour prior to extraction with 20 mL of methanol overnight.

6.2.10 Investigation of the loss of 3-CA due to enzymatic activity

6.2.10.1 Effect of spiking time on the recovery of 3-CA from potato juice

To investigate the effect of the enzymatic activity on the extraction of 3-CA from potato samples, the effect of spiking time on the recovery of 3-CA from potato juice was investigated. Three potato tubers (~ 250 g) were cut and blended using a household food processor to produce a homogenised potato juice which was filtered by vacuum using Whatman filter paper No. 54. 2 mL of the filtered juice was spiked with 100 µL of 200 µg/mL of 3-CA spiking solution and left for a range of different time periods (0, 2, 5, 10, 20, 30 and 60 minutes) prior to overnight soaking extraction with 18 mL of methanol solution containing 1 µg/mL IPC. Two different 3-CA spiking solutions were prepared in each of methanol and water. A control blank was prepared from the nonspiked potato juice.

6.2.10.2 Preventing the enzymatic reaction in the potato juice

An experiment was designed to study the inhibition of the enzymatic oxidation in potato juice and its effect on the extraction of 3-CA. 400 g of whole potato (Cosmos organic) were blended. After filtration with a Whatman filter paper No. 54, the juice was divided into four parts. 1 g of the reducing agents sodium dithionite and ascorbic acid were added to two 50 mL portions of juice and left to stand for 15 minutes. Another 50 mL of juice was placed into a 100 mL screw jar without a lid and immersed in a water bath at 90 °C for 10 – 15 minutes and finally left to cool. The remainder of the juice was left without treatment as a control. All the treated juices and the control were refiltered by vacuum filtration using GF/C membrane filter paper. After filtration, 2 mL from each juice (n = 3) was taken and spiked with 100 µL of 200 µg/mL solution of 3-CA in methanol for 1 hour. This was followed by the extraction process where 18 mL of a 1 µg/mL IPC solution in

methanol was added as the extractant and left for overnight soaking. In each case, triplicates of blank controls of nonspiked juice were prepared.

6.2.10.3 Preventing the enzymatic reaction in potato peel

To examine the role of enzymatic oxidation inhibitors on the extraction of 3-CA from potato peel, spiking solutions of 100 µg/mL of 3-CA were prepared in each of ascorbic acid solutions (1, 5, 10 and 15 %), citric acid solutions (1, 5, 10 and 15 %), a combination of ascorbic acid and citric acid (10% and 15% respectively), 1 M sulphuric acid and deionised water as a control. After spiking chopped peel with 200 µL of each of these solutions, the overnight soaking extraction method as described in Section 6.2.1.3 was followed (five replicates were included in each case). The Osprey potato variety (untreated with CIPC) was used in this experiment.

6.2.11 Investigation of pH effect and ion exchange on extraction of 3-CA from the potato peel

In order to enhance the extraction of 3-CA, experimental trials were carried out using extracting solutions of acid, base and salt materials including 0.25 M sodium hydroxide, 0.3 M ammonia, 1 M sodium carbonate, 0.4 M lithium acetate, 0.3 M ammonium acetate, different concentrations (0.1, 0.9 and 1.8 M) of glacial acetic acid and 1 M sulphuric acid. These materials were added into the extractant (methanol) containing an internal standard with the exception of sodium hydroxide and sodium carbonate (which were prepared in water). Chopped peel ($n = 5$) was spiked with a 3-CA solution in methanol whereas the spiking solution of 3-CA in water was used in the case of sodium hydroxide and sodium carbonate, then followed by overnight extraction (see Section 6.2.1.3). The pH of the extract was adjusted (pH ~ 2 – 8) using 0.5 M and 1 M NaOH and concentrated acetic acid (17.5 M). Several organic potato varieties were used in this work, namely Carling, Osprey, Nicola and Sante.

6.2.12 Influence of acidity on the extraction of 3-CA

6.2.12.1 Influence of acidity on chromatographic separation

This part of the research involved studying the effect of acidity on the chromatographic separation of 3-CA by analysing standard solutions of 1 µg/mL of 3-CA and IPC in methanol containing different concentrations of acetic acid (0, 0.5, 2.5, 5 and 10%). Additionally, a standard solution of 1 µg/mL of 3-CA and IPC was prepared in an

extracting solution of 1 M H_2SO_4 in 50% methanol at ambient temperature (which was used to extract the spiked and nonspiked peel) (see Section 6.2.12.2). These solutions were analysed after adjusting the pH ($\sim 2 - 8$) with 0.5 or 1 M NaOH.

6.2.12.2 Extraction of 3-CA using sulphuric acid in different percentages of methanol

Sulphuric acid as a strong acid combined with methanol was used to extract 3-CA from the spiked potato peel. 1 M of this acid in different percentages of methanol (0, 10, 25, 50, 75, 90 and 100%) was prepared. 20 mL of each concentration was used to extract the chopped peel ($n = 5$) after spiking with a water solution of 3-CA. 2 mL of the extract was neutralised with a suitable volume of 1 M NaOH and made up to 5 mL with methanol prior to transfer into an HPLC vial for analysis. The standard solution was prepared from the same spiking and extracting solutions and neutralised with 1 M NaOH. This experiment used two varieties of potato: Nicola and Maris Peer.

6.2.12.3 Influence of temperature on the extraction of 3-CA

To assess the influence of temperature on the extraction recovery of 3-CA from spiked peel using 1 M sulphuric acid in 50% methanol, a range of temperatures (ambient, 22, 50 and 70 °C) were tested. This experiment involved using two spiking solutions of 3-CA prepared in each of methanol and water. The procedure in Section 6.2.1.3 was applied using 1 M sulphuric acid in 50% methanol as the extractant and replicates were sustained at each of the temperatures for a period of 16 hours. All analyses were performed in triplicate as was the nonspiked peel (control blank). The pH of the extract was adjusted as above prior to analysis.

6.2.12.4 Influence of extraction time on the extraction of 3-CA

To establish the optimal time for extraction of 3-CA, an experiment was conducted to determine the effect of extraction time on the yield of 3-CA extracted from potato peel ($n = 3$) spiked with methanol solution of 3-CA and extracted using 1 M sulphuric acid in 50% methanol solution containing 2 $\mu\text{g/mL}$ IPC. The replicates were kept in the incubator at 50 °C for a range of different time periods (2, 6, 12, 18 and 24 hours). Prior to analysis by HPLC, the pH of the extract was adjusted with 1 M NaOH. Three replicates of nonspiked peel as control blank were also prepared. The Osprey variety of potato was used for this experiment.

6.2.12.5 Influence of acidity on the degradation of CIPC

The degradation of CIPC by acid hydrolysis under the experimental conditions of the extraction was investigated. A simple experiment was carried out by preparing a solution of 10 µg/mL CIPC using the same extracting solution mixture of 1 M sulphuric acid in 50% methanol (containing 10 µg/mL IPC). This solution was kept at the extraction temperature of 50 °C for 24 hours. The chromatogram analysis of this solution was compared with a mixed standard solution of 10 µg/mL of 3-CA, IPC and CIPC prepared in a mixture of 1 M sulphuric acid in 50% methanol at ambient temperature.

6.2.13 Application of the proposed method for the determination of the residues of 3-CA and CIPC in stored potato tubers treated with CIPC

The new extraction method (see Section 6.3.14) using a mixture of 1 M H₂SO₄ in 50% methanol at 50 °C for 24 hours was tested on 20 potatoes tubers treated with CIPC from a commercial potato store to determine the residues of both CIPC and 3-CA. The new method was then compared with the existing method for extraction of CIPC described in Chapter five (summarised in Section 5.3.5.2) and in addition, the new method was performed under ambient temperature conditions. The analysis was performed using two HPLC systems namely the autosampler SpectraSERIES UV100 (system employed for the new extraction method) and the Hitachi DAD HPLC (to check the purity of the peaks).

6.2.14 Effect of fogging temperature and the number of CIPC applications on the residue levels of 3-CA and CIPC in stored potatoes

The new extraction method (see Section 6.3.14) was used to investigate the effect of fogging temperature and the number of CIPC applications on the residue levels of 3-CA and CIPC in potato samples taken from two UK commercial stores at the start of the storage season 2010 – 2011. These potatoes had been treated once and the application details for these stores are summarised in Table 6:1. Later, further samples from store 2 were analysed after a second application of CIPC.

Table 6:1. Application information for the potato stores that supplied the potato samples.

Store	Type	Fogging temperature	Application rate
Store 1	Bulk	450 °C	14 g/tonne
Store 2	Box	270 °C	12 g/tonne

6.3 Results and discussion

As recent demand from the EU in 2009, was that the residues of the potato sprout inhibitor CIPC and its metabolite 3-CA in potato tubers should be monitored in terms of a MRL of CIPC which must include both of CIPC and 3-CA. This is an important issue for human consumption and therefore the potato industry as a whole. Therefore, it is essential to develop and validate an analytical method for the simultaneous determination of the residue levels of both CIPC and 3-CA in potato samples. For this purpose, the previous work in Chapter five involved developing a new method that utilised a simple and economical extraction procedure with high sensitivity based on methanol-soaking for at least 16 hours before being analysed on an HPLC-UV. Although a high extraction efficiency of CIPC was achieved from the potato samples, this method obtained a very low recovery for 3-CA of less than 10% at spiking level 8 µg/kg of potato peel. As it is polar and has a high water solubility, there was no concern for the adsorption of 3-CA onto laboratory glassware. The non-adsorption of 3-CA on laboratory glassware was proved in previous work (see Section 4.3.2.5). Therefore, the low recovery represents low extractability from the potato tissue. So, considerably more investigation was required to enhance the extraction efficiency of 3-CA from potato samples and establish an optimised extraction method with acceptable recovery. The work in this chapter set out to focus on this problem.

6.3.1 Influence of potato variety on the extraction 3-chloroaniline

In the UK, there are more than 80 commercial varieties of potato grown under different environmental conditions and requirements in various production areas. These varieties have different characteristics including tuber shape, internal structure, moisture, texture, nutrient content and skin which vary in their colour and composition (Seefeldt et al., 2011; Ortiz-Medina et al., 2009).

In order to investigate the role of the potato variety on the extraction of 3-CA from spiked peel, four varieties of organic potatoes (Maris Peer, Valor, Orla and Sante) were used. These potatoes had not received any pesticide application. All four varieties have a smooth

and cream to light yellow colour skin with cream flesh. Peel samples from these varieties were spiked with 3-CA and extracted using the methanol solution (see Section 6.2.1.3). Table 6:2 presents the recovery results obtained for HPLC analysis of the extract of 3-CA from the spiked peel using the existing CIPC extraction method in Chapter five.

Table 6:2. The recoveries of 3-CA from the spiked peel of different potato varieties at a concentration of 1 µg/mL.

Variety	Recovery %	RSD% (n = 5)	Tukey test
Maris Peer	5	9.8	a*
Valor	8	13.0	b
Orla	9	8.9	b
Sante	10	5.6	c

a*: Different letters refer to a significant difference ($p < 0.05$) using Tukey HSD

It is apparent from Table 6:2 that very low recoveries of 3-CA ($\leq 10\%$) were obtained for the extraction of the spiked peel for all four varieties of potato. The differences between the means of the recoveries for 3-CA were examined using analysis of variance (ANOVA) with a Tukey HSD test. This test showed a significant difference ($p < 0.05$) among all the varieties with exception of between Valor and Orla which were the same.

Overall, although the recovery varied between these varieties the results of this investigation showed generally very low extraction recovery for 3-CA.

6.3.2 Influence of solvent on the extraction 3-chloroaniline

Extraction of organic compounds from environmental samples using solvents is affected by several factors. Selection of the appropriate solvent is the most important factor in being able to extract the target compound from the sample matrix. The solvent selected depends on the nature of the compound and the sample matrices from which the target compound of interest is being extracted. In particular, the solvent used should have a polarity similar to the target compound in order to control its solubility. Generally, for the extraction of aromatic amines, polar organic solvents are more suitable than non-polar. However, in a few cases some interferences may be encountered due to the co-extraction of other compounds from the sample which can cause chromatographic problems and difficulties in distinguishing the target compound (Zhu et al., 2002; Yazdi and Es'haghi, 2005).

In order to investigate the role of the solvent on the extraction efficiency of 3-CA from spiked potato peel samples, several organic solvents were examined by applying both the soaking and Soxhlet extraction methods. The effect of the solvent used as a spiking solution on the extraction was also tested using various solvents. The extraction efficiency was calculated as percent recovery following analysis of the extract of the spiked peel as shown in Table 6:3.

Table 6:3. The recovery of 3-CA and RSD% values for spiked potato peel using different spiking and extracting solvents at a concentration of 1 µg/mL.

Spiking solvent	Extracting solvent	Extraction method	Recovery%	RSD% (n = 5)
Acetonitrile	Acetonitrile	Soaking	11	7.8
Methanol	Methanol	Soaking	10	20.8
Water	Methanol	Soaking	29	5.7
Water	Methanol	Soxhlet	34	15.5
Water	Formic acid	Soxhlet	31	9.1
Water	Dichloromethane	Soxhlet	ND*	-
Hexane	Hexane	Soxhlet	23	25.3
Hexane/ Na ₂ SO ₄	Hexane	Soxhlet	ND	-

ND*: No peak detected

These data are quite revealing in several ways. Firstly, all the solvents used to extract 3-CA yielded low recoveries for 3-CA. However, it is apparent from the table that spiking solvent had an impact on the recovery value (this will be discussed in detail later in Section 6.3.5). Spiking the peel with a water solution of 3-CA yielded recoveries that were a little higher than those obtained for spiking with organic solvents (with exception of dichloromethane). It may be that the 3-CA dissolved in an organic solvent penetrates more deeply into the potato peel than when dissolved in water and therefore the subsequent extraction is more difficult. Secondly, when using sodium sulphate with hexane in the spiking procedure there was no peak for 3-CA, whereas using hexane alone, the recovery was 23%. This might be due to the evaporation of 3-CA during grinding of the spiked peel with Na₂SO₄ in the uncovered mortar, which was left for 1 hour prior to the Soxhlet extraction. It was thought that removing the water from the spiked potato peel using a drying agent (sodium sulphate) would improve the recovery, however, the results show the opposite. Taking these two findings together, the spiking method had an effect on the

extraction of 3-CA. Thirdly, the polarity of the solvent plays also an important role in the extraction. Results of the Soxhlet extraction showed similar recoveries with methanol (34%) and formic acid (31%) whereas no peak for 3-CA was detected in the extract using the less polar solvent dichloromethane. The immiscibility of dichloromethane with the aqueous spiking solution of 3-CA could have an effect on the extraction. Lastly, in spite of using different extraction procedures, no big difference was found between soaking (29%) and Soxhlet extraction (34%) when using the same spiking solvent of water and extracting solvent of methanol.

The main finding to emerge from this investigation is that none of the organic solvents used gave satisfactory extraction of 3-CA from the potato peel. However, there are a number of important factors that may have an effect on the extraction recovery which need to be investigated such as the extraction method, the polarity of the extractant, spiking procedure and interaction of 3-CA with the potato sample.

6.3.3 Influence of extraction method on 3-chloroaniline recovery

The extraction of organic compounds from environmental matrices is most commonly performed using traditional liquid-solid extraction methods. The most simple and common of these methods range from soaking in solvent to those that require heating like Soxhlet extraction and those which use agitation, i.e. shaking or sonication bath (Dean, 1998). The extraction time for these methods varies from minutes up to 24 hours. To accelerate the extraction process, high temperature and high pressure of the extractant can be utilised such as is done with both pressurised liquid extraction (PLE) and supercritical fluid extraction (SFE) (Morales-Munoz et al., 2003). Additionally, energy sources such as shaking or ultrasounds can accelerate sample extraction. Using ultrasonic treatment can generate extreme temperatures and pressures as a result of collapsed gas bubbles that lead to enhanced chemical reactivity and rapid extraction (Hua and Hoffmann, 1997). Mechanical shaking is used to facilitate and accelerate the extraction when the analyte is very soluble in the extraction solvent and the sample matrix is finely ground material.

The aim of this part of the work was to evaluate the extraction efficiency of 3-CA from spiked peel samples using different extraction methods. Five extraction methods using methanol as the extractant were applied including soaking overnight, sonication, heating, stirring and Soxhlet extraction. These extraction methods were selected because they are commonly used methods in the University of Glasgow laboratory to extract CIPC residues from potato samples.

Table 6:4 shows the experimental recoveries obtained from the analysis of the extracts obtained from all five extraction methods tested.

Table 6:4. Recoveries, RSD% values and statistical analysis for 3-CA extraction using different extraction methods.

Extraction method	Recovery %	RSD% (n = 5)	Tukey test
Soaking (~ 16 hr at ambient temp)	10	23.1	a*
Sonication (30 min at 50 °C)	13	16.5	a
Heating (45 min at 50 °C)	14	9.9	a
Stirring (1 hour at ambient temp)	11	46.3	a
Soxhlet (3 hours)	11	11.9	a

a* : Same letters refer to non-significant difference ($p > 0.05$) Tukey HSD

Despite the differences in the procedure and the extraction time of these five methods in addition to the possible effects of many factors such as temperature, stirring and heating, the recoveries in Table 6:4 show similar poor extraction for 3-CA. It is also apparent from the ANOVA (one-way) Tukey test results that these recovery results were not statistically different ($p > 0.05$). This experiment provides strong evidence that the low recovery of 3-CA from spiked peel is not due to the extraction procedures and the reason for this is not clear but it may have something to do with spiking procedure and the subsequent interaction between 3-CA and potato peel constituents.

6.3.4 Influence of spiking time on the extraction of 3-chloroaniline from potato peel

In order to study the effect of the spiking time on the extraction of 3-CA, chopped peel ($n = 5$) was spiked with a methanol solution of 3-CA and left to stand for different periods of time (0, 2 – 3, 5 and 60 minutes) prior to extraction, followed by 16 hours soaking in methanol (see Section 6.2.1.3). It can be seen from Figure 6:3 that there is a clear trend of decreasing recovery of 3-CA with increasing time of contact between the spiking solution and the potato peel sample prior to extraction.

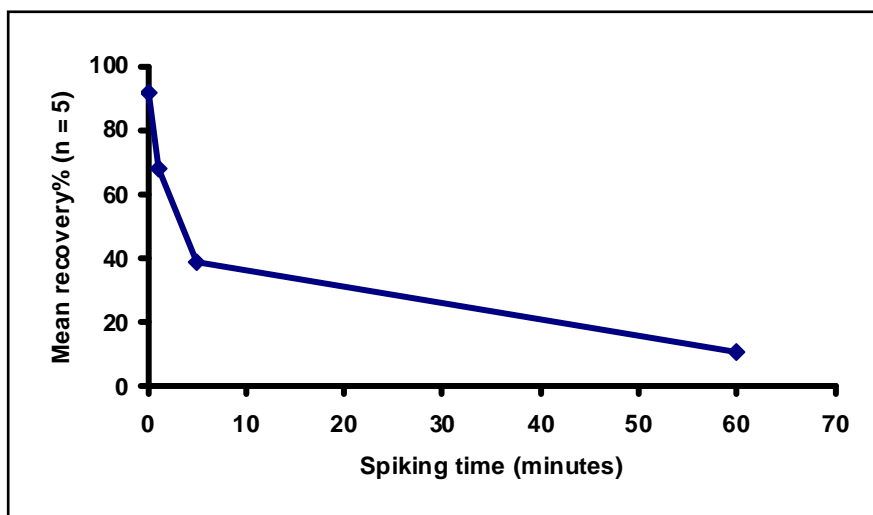


Figure 6:3. The effect of the spiking time on the recovery of 3-CA from potato peel.

In this figure, the highest recovery value was obtained at spiking time zero minute when both 3-CA solution and the extractant were added simultaneously to the potato peel (i.e. no standing time after addition of the spike). This meant there was no direct contact of 3-CA with the peel before the extractant was added. Due to its polarity, 3-CA remains in the polar solvent methanol more easily than penetrating into the potato peel (and therefore reduces the loss of 3-CA). In contrast, the remaining recovery values shown in Figure 6:3 decrease because the spiking solution of 3-CA was added: firstly in a small volume of high concentration (100 μ L 200 μ g/mL) to the peel and secondly, it was left to stand for selected time period prior to addition of the extractant, this meant there was greater opportunity for the 3-CA to penetrate deeper into the potato peel.

Overall, it can be concluded that the longer the time between addition of the spike and addition of the extractant, the more difficult it becomes to extract 3-CA from the peel.

In reviewing the literature, some experiments were conducted to examine the effect of spiking time on the recovery of certain aromatic amines (4-chloro-o-toluidine, 2-naphthylamine, 4-aminobiphenyl and benzidine) from finger-paints using supercritical fluid extraction (SFE) (Garrigos et al., 2000). In this study, samples were spiked with the aromatic amines and stored for between 2 – 24 hours prior to extraction with methanol. It was observed that the recovery decreased from the time zero experiment to the 2 hour experiment and a further decrease in recovery was found with the 24 hour experiment. The authors speculated that this was due to the adsorption of the aromatic amine into the paint matrix.

6.3.5 Influence of spiking solvent on the extraction 3-CA

This experiment was conducted to investigate the effect of the spiking solution on the extraction of 3-CA from spiked peel. Solutions of 3-CA were prepared in different solvents with which to spike the potato peel prior to extraction. Again, the samples were left to soak in a methanol solution for 16 hours. The recovery results obtained from the HPLC analysis of the extract are shown in Table 6:5

Table 6:5. Recoveries of 3-CA from spiked peel using different spiking solvents and a concentration of 1 µg/mL.

Spiking solvent	Recovery %	RSD% (n = 5)	Tukey test
Water	29	5.7	a*
Methanol	10	20.8	b
Chloroform	16	16.3	c
Dichloromethane	6	12.2	b

*Different letters refer to a significant difference ($p < 0.05$) Tukey HSD

As can be seen from the table all the solvents used to prepare the spiking solution of 3-CA resulted in poor extraction from the spiked peel, using the same extractant methanol. The differences between the means of the recoveries were tested using analysis of variance (ANOVA) with a Tukey HSD test. The results showed significant differences ($p < 0.05$) between these solvents, with the highest recovery obtained where water was used for the spiking solution. No difference was found between methanol and dichloromethane, with both extracting a similar amount of 3-CA.

As mentioned before in Section 6.3.2, a higher recovery was produced when using water solution of 3-CA spiking; this could be related to the high polarity of water and its incompatibility with the hydrophobic nature of potato peel, relative to the other solvents such as dichloromethane (which is the least polar in this group of solvents).

6.3.6 Extraction of 3-chloroaniline from spiked potato samples

A series of experiments were carried out to examine the extraction of 3-CA with methanol soaking from spiking fresh peel, treated potato peel samples and different parts of the tuber with a methanol solution of 3-CA (as described in Section 6.2.1.3). The recovery results are presented in Table 6:6.

Table 6:6. Recoveries of 3-CA from spiking different potato samples at concentration of 1 µg/mL.

Spiking materials	Recovery %	RSD% (n = 5)
Fresh peel	10	17.5
Dried peel	86	6.3
Dried peel rewetted	36	3.3
Peel treated with chloroform	6	7.8
Peel treated with methanol	86	2.3
Brown surface of peel (periderm)	26	19.0
White surface of peel (cortex)	20	22.4
Skin	4	13.6
Cortex	20	17.1
Pith	49	7.5

The most striking result to emerge from this table is that the recovery of 3-CA from these different potato samples varied from between 4 to 86%, thus giving poor to acceptable extraction recoveries despite using the identical spiking concentration and extraction procedure. These differences may be due to different binding strength of 3-CA on the surfaces of these potato samples.

Potato peel like many fruits and vegetables contains water; the percentage of water is approximately 90% (see Section 2.1.9). Assessment of the role of drying potato peel on the recovery of 3-CA by drying in an oven overnight at 100 °C gave high recovery results (86%) with a good RSD% compared with the low recovery obtained when spiking fresh peel (10%). However, spiking the dried peel after rewetting with deionised water showed less recovery (36%) than for extraction of the spiked dry peel but this was more than 3 time the recovery obtained with the fresh peel. From these results it can be concluded that the presence of water in the peel before addition of the spike is an extremely important factor in the mechanism that reduces 3-CA extractability.

Another consideration to be taken into account regarding the composition of the peel that may have an effect on the extraction of 3-CA is that potato peel contains several compounds, such as polyphenols and carotenoids (Al-Weshahy and Rao, 2009). Approximately 50% of phenolic compounds are located in the potato peel and adjoining layers and their concentration decreases from the outer layers toward the centre of the flesh (Friedman, 1997). Therefore, an experiment was conducted by treating the peel with

methanol, using Soxhlet refluxing for ~ 3 hours, until the solvent in the extractor became clear in an attempt to extract these phenolic compounds from potato peel. Methanol was used in order to avoid incompatibility as all spiking, extracting and standard solutions were dissolved in methanol. Methanol and ethanol were found to be the best organic solvents to extract phenolic compounds from plant materials due to their high polarity and good dissolution power for these compounds (Mohdaly et al., 2010). The recovery from spiking the treated peel with methanol showed high recovery (86%). This finding was unexpected and suggests that loss of some substrates or removal of water from the treated peel through Soxhlet extraction considerably increases the extractability of 3-CA.

It was thought that the waxes associated with the potato peel might also be responsible for the low extractability of 3-CA. These layers of wax consist of many components, such as hydrocarbons, wax esters, free fatty acids, free fatty alcohols and other unknown compounds (Espelie et al., 1980). Wax layers are embedded in an extracellular matrix and can be removed by dipping in an organic solvent like benzene or chloroform for a short period of time (Kolattukudy, 1965). Therefore, an attempt was made to remove the entire wax layer by dipping the potato tuber in chloroform. However, in this study, extraction of the wax from the potato tuber peel did not enhance or improve the recovery of 3-CA (6%). It should be noted that chloroform can remove the wax from the peel but is not able to withdraw moisture which might have had an adverse effect on the extraction 3-CA as mentioned above. Removing the wax from the peel was strong evidence that the wax composition has no effect on the binding of 3-CA to the potato peel surface.

Potato tubers are composed of five main physiologically distinct tissues (see Figure 6:1), namely the skin or periderm (the coloured outer layer of a potato tuber), the cortex (the area between the skin and vascular ring), the vascular ring, the outer medulla and the inner medulla (pith) which is the more translucent and wetter part in the centre of the potato tuber (Woolfe, 1987). The percentage contributions of these layers are considerably varied of the whole tuber, due to difficulties of defining the boundaries precisely and to differences between potato tubers. The dry matter components such as carbohydrates, soluble protein, antioxidants, vitamins and minerals are not evenly distributed in potato tuber tissues (Ortiz-Medina et al., 2009; Li et al., 2006; Shepherd et al., 2007; Thomas and Delincee, 1979). For example, the concentrations of glycoalkaloids and phenolic compounds in the skin are higher than in other layers of the potato such as the cortex and the pith (Ponnampalam and Mondy, 1983; Shepherd et al., 2007). When a potato is peeled, even very thinly, the resulting peel has two distinct surfaces the periderm (brown surface)

and the cortex (white surface). As can be seen from Table 6:6, spiking the two surfaces led to little difference in 3-CA recovery. The peel layer may be thin enough to allow the methanol spiking solution to penetrate from either surface. However, when samples of skin, cortex and pith were tested the recovery of 3-CA showed a clear trend of increasing recovery; 4%, 20% and 49% respectively. It seems possible that these different recovery results are due to the difference in the composition of these layers within the potato tuber.

In summary, all the attempts above showed penetration of 3-CA into the potato samples depending on the nature of the peel (fresh, dried, treated with solvent), the component layers of the potato, spiking solvent and spiking time. At this stage, any mechanism needs to be interpreted with caution but there is strong evidence that the presence of water in the peel is important. Possibly as the medium in which the mechanism occurs. Mechanisms hypothesised to explain the fate of 3-CA and its subsequent poor extraction from spiked peel include:

1. Volatilisation

3-Chloroaniline has a high vapour pressure. Loss of 3-CA during spiking due to volatilisation seems unlikely in a sealed system; however it will be tested.

2. Chemical reaction with potato components

Decomposition of 3-CA may occur due to chemical reaction with components in the potato. As a Lewis base and in the presence of water, a typical reaction of the aromatic amine can be a nucleophilic addition with a carbonyl group present in potato skin constituents resulting in a compound with a C=N functional group, which is called an imine (Schiff base).

3. Enzymatic reaction

Decomposition of 3-CA may occur due to enzymatic activity in the potato cell either by acting on 3-CA directly or by oxidase enzymes producing products (probably quinone) which can interact chemically with 3-CA (e.g. Schiff base).

4. Ion exchange related to pH

Binding of 3-CA to potato peel may be based on an ion exchange process. This mechanism was speculated upon, on the basis that the negative charge (e.g. COO^-) of the potato cell wall may interact electrostaticly with 3-CA by ion exchange.

Considering these suggestions, therefore, further experiments were undertaken to investigate these possibilities in more detail.

6.3.7 Investigation of 3-CA volatilisation losses during spiking

3-Chloroaniline is a volatile compound at ambient conditions because of its relatively high vapour pressure of 0.066 mm Hg (8800 mPa) at 25 °C (SRC, 2011).

Park (2004) calculated a theoretical saturated vapour concentration for 3-CA in equilibrium with liquid 3-CA as 468 µg/L at 25 °C. This theoretical value could be 47 µg in the 100 mL headspace of the sealed spiking jar compared to an addition of only 20 µg of 3-CA spiked on the potato peel in the present study. That means if volatilisation occurs all of the 3-CA spiked could be lost to headspace. Park (2004) also compared the volatility of 3-CA in a static system with other potato sprout inhibitors including 1,4-DMN and tecnazene. The experiments were designed by adding each chemical to the bottom of a sealed jar, which was then held at a constant temperature. After 24 hours during which equilibration was reached, samples were withdrawn from the headspace of each jar using a disposable needle and syringe, which was then plugged with a Teflon septum prior to injection onto a packed column GC. The measured saturated vapour concentrations of 3-CA in µg/L were 357 ± 82.4 at 30 °C and 93 ± 35.4 at 20 °C. The values for 3-CA were much higher than for both of 1,4-DMN and tecnazene due to the higher volatility of 3-CA.

6.3.7.1 Using empty jars without peel

In this study, the possible volatilisation of 3-CA during spiking was measured by adding a spiking solution of 3-CA to an empty sealed jar with no potato tissue, allowing it to stand for 1 hour prior to overnight extraction. The recovery results of five replicates are shown in Table 6:7.

Table 6:7. Recovery values for spiking an empty jar at two spiking levels using two solvents.

3-CA spike	Spiking solvent	Extracting solvent	Recovery %	RSD (n = 5)
2 µg	Methanol	Methanol	93	11.2
	Acetonitrile	Acetonitrile	99	7.9
20 µg	Methanol	Methanol	100	2.3
	Acetonitrile	Acetonitrile	101	3.7

It is apparent from the table that under the conditions used to spike the potato tissue, there is negligible loss of 3-CA by volatilisation.

6.3.7.2 Use of Tenax traps in collection system

The use of solid adsorbents is often one of the trapping methods used to collect volatiles from potato. Sampling tubes are filled with these solid adsorbents to concentrate traces of volatile organic compounds from air (Russell, 1975). Passing a measured volume of air through an adsorption tube at a controlled rate collects the vapour from the sample on to the packing material. Recently, this method was used for headspace analysis of the potato sprout inhibitor 1,4-DMN (Oteef, 2008). The Tenax trap is a glass tube packed with solid adsorbent of porous polymer based on 2,6-diphenyl-p-phenylene oxide which offers a high thermal stability for gas chromatography separation (Ponder, 1974).

In this work, a further test of volatilisation of 3-CA from spiked peel under controlled conditions was performed based on the collection system used by Oteef (2008) for collection of potato volatiles as shown in Figure 6:2. The system was set up where a stream of nitrogen gas was passed through a bed of potato peel (25 g) spiked with 3-CA and then through a Tenax column to trap any 3-CA released into the headspace. This experiment was run for 24 hours, after which the Tenax trap was eluted with 10 mL of acetonitrile. Even under these more extreme conditions, the chromatogram obtained from HPLC analysis of the Tenax trap elution showed no peak of 3-CA compared with a standard solution as shown in Figure 6:4.

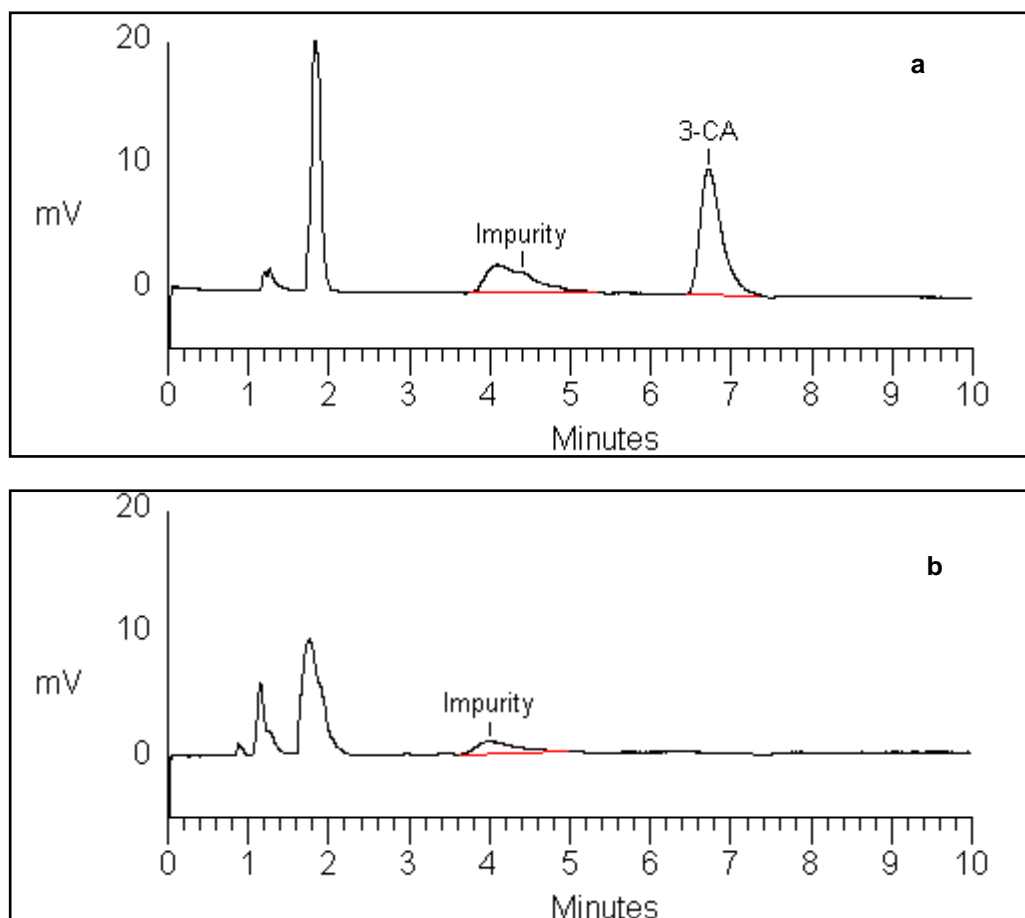


Figure 6.4. Chromatograms of analysis of: a- 1 µg/mL standard solution of 3-CA and b- the acetonitrile eluate from sampling Tenax trap.

It can be concluded from this experiment that the reason for the low recovery of 3-CA from spiked peel is not related to the volatility of this compound (under these experimental conditions) even although it is known to have a high vapour pressure.

6.3.8 Investigation of the loss of 3-CA by reaction with different potato chemical components

3-Chloroaniline as an aromatic amine and is a weak base. Generally, reactions of aromatic amines are strongly reactive in electrophilic aromatic substitution because of the electron – donating effect of the amino group. The formation of a Schiff base involves the reaction between an aromatic amine and either an aldehyde or a ketone. It was reported that the resistance of the residue of chloroanilines to solvent extraction from soil strongly suggests a covalent binding of the nucleophilic amino functional group to electrophilic sites of a carbonyl group or quinoidal ring of humic compounds in soil (Hsu and Bartha, 1976; Hsu and Bartha, 1974; Adrian et al., 1989; Weber et al., 2001).

6.3.8.1 Reaction with glucose

The main purpose of this work was to investigate the possibility of reaction of 3-CA with the chemical constituents of the potato. It has been speculated that the amino group of 3-CA has a tendency to react with the carbonyl group in terms of a Schiff base reaction with glucose (Harry Duncan, personal communication) which is one of the major reducing sugars and an important carbohydrate found in potato (100 mg/100g) (Martin and Ames, 2001). Table 6:8 shows recovery values for spiking different weights of glucose with 3-CA for 1 hour prior to overnight soaking extraction with 20 mL of 1 µg/mL IPC in methanol.

Table 6:8. Recoveries and RSD% values from spiking different weights of solid glucose mixed with water.

Wt. (g)	Water (mL)	Methanol extractant (mL)	Recovery%	RSD% (n = 5)
1	-	20	97	0.6
0.5	1	20	93	2.9
1	1	20	72	5.2
3	1	20	56	10.6

As can be seen from the table there is a clear trend of decreasing recovery with increasing weight of glucose (in the presence of water). Therefore, it seems possible that the observed reduction in recovery could be attributed to reaction of the amino group of 3-CA with the aldehyde group of glucose. Hydrogen bonding may also be suggested to play a vital role between these two groups. It was mentioned that acetic acid can catalyse the reaction between aniline and glucose to produce a brown coloured material glucose-anilid. This reaction is more rapid in acidic solutions than with glucose and aniline alone (Cameron, 1926).

6.3.8.2 Reaction with other potato chemical components

To investigate other possibilities of the reaction of 3-CA with other potato components, several chemicals which are present at high concentrations in potato were selected:

Chlorogenic acid: is a major phenolic compound which constitutes about 90% of the total phenolic content of potato tuber; (its concentration can range from 1 – 2 mg/100g dry weight) (Malmberg and Theander, 1985; Singh et al., 1998).

Caffeic acid: is a polyphenolic substance present in potato (10 – 41 mg/100g DM) (Lisinska and Leszczynski, 1989), it is found along with chlorogenic acid, to be present at

higher concentrations in potato peel than in potato flesh. Both play an important role in enzymatic browning and act as antioxidants (Rodriguez De Sotillo et al., 1994; Hayase and Kato, 1984).

Asparagine: is natural amino acid (amide of aspartic acid) and is present in the highest amount in potato (93.9 mg/100g) (Martin and Ames, 2001). It is thought that reaction of asparagine with reducing sugars may be responsible for the formation of acrylamide during potato frying (Zhu et al., 2010).

Citric acid: is a major organic acid naturally present at high levels in potato tubers as compared with other acids. It plays an important role as an antioxidant in the oxidative process that decreases the tendency of boiled potatoes to darken (Wichrowska et al., 2009; Lisinska and Leszczynski, 1989).

Aspartic acid: is one of the most abundant amino acids present in potatoes (1990 mg/100g DW) (Zhu et al., 2010).

Glutamic acid: is a natural amino acid considered to be a flavour enhancer that is present in potato tuber (2180 mg/100g DW)(Zhu et al., 2010).

Arginine: is another amino acid found in potato, but at lower concentrations relative to other amino acids.

Tyrosine: is an amino acid present in potatoes in relatively high concentrations (575 mg/100g DW) and plays an important role in the browning of potato during the oxidation process that occurs when the potato tissue is damaged (Field et al., 1987).

Malonic acid: is an organic acid reported to be present in potato tubers, but in lower quantities, organic acids in general affect the acidity of potato juice (Lisinska and Leszczynski, 1989).

Ascorbic acid: is a natural organic compound found at 3 mg/100g dry weight in new potato and acts as an antioxidant in potato and is the acidic form of vitamin C (Davey et al., 2000).

In this study, these compounds were added to a 3-CA solution in water and left for 1 hour. Methanol was added and the residual 3-CA was measured after standing overnight to stimulate the extraction. The recovery results of 3-CA are presented in Table 6:9.

Table 6:9. Recovery values for 3-CA after contact with solutions of different potato chemical components.

Material	3-CA Recovery%
Chlorogenic acid	108
Caffeic acid	110
Asparagine	98
Citric acid	104
Aspartic acid	102
Glutamic acid	108
Arginine	108
Tyrosine	102
Malonic acid	99
Ascorbic acid	99

It is apparent from Table 6:9 that high recoveries of 3-CA were obtained (in the range 98 – 110%) from extraction of its mixed solution with each compound. These recovery results indicated that no reaction occurred between 3-CA and these chemicals under the experimental conditions.

6.3.9 Investigation of the loss of 3-CA due to enzymatic activity

It was suggested that peroxidase enzymes may be involved in the degradation and binding of chloroaniline-based pesticide residues in soil (Fletcher and Kaufman, 1980). Chloroanilines can interact with peroxidase enzymes with the formation of polymeric compounds with both anil- and quinoid type bonds (Hsu and Bartha, 1976; Hsu and Bartha, 1974; Adrian et al., 1989). The formations of these quinoid bonds require an initial oxidation of the substituted aromatic ring by the peroxidase enzymes. The reaction products of chloroaniline may have some affinity to bind to humic materials in soil (Fletcher and Kaufman, 1980) or possibly by comparison with lignin in plant.

One example of a common enzyme in potatoes is polyphenol oxidase (PPO) which has a copper ion bound in the active site and catalyses the oxidation of polyphenolic compounds by oxygen to the corresponding reddish-brown of o-quinones (Girelli et al., 2004; Kim,

1995). These products are highly reactive and can non-enzymatically auto polymerise to yield an insoluble black-brown coloured melanin pigment (Eicken et al., 1998; Busch, 1999). This phenomenon is often called a browning reaction. The most important factors affecting enzymatic browning are the concentration of active enzyme PPO, the concentration of phenolic compounds, pH, temperature and the presence of the oxygen in the tissue of fruits and vegetables (Martinez and Whitaker, 1995; Chutintrasri and Noomhorm, 2006).

There are several possible explanations for the role of enzymatic oxidation in the poor extraction of 3-CA. PPO is a highly effective enzyme, therefore its presence in the potato might be responsible for enzymatic breakdown and oxidation of 3-CA directly. Another possibility is that of 3-CA reacting with the o-quinone products of the PPO enzyme activity. Thus, these potential reactions of 3-CA in potato tissue may well lead to a difficult extraction and a subsequent low recovery. The rate of these reactions can be influenced by various factors such as the concentration of PPO, quinone and 3-CA, contact time, pH and temperature. Moreover, the potato variety and its moisture presence have potential effect on the extraction of 3-CA as discussed in Sections 6.3.1 and 6.3.6.

The following series of experiments investigated the ability of the enzymes present in potatoes to catalyse possible 3-CA breakdown reactions in potato tissue by using potato juice to study the processes.

6.3.9.1 Effect of spiking time on the recovery of 3-CA from potato juice

Since potatoes contain a high percentage of water, it is easy to extract juice using a home blender. Blending potato results in the juice instantly developing a brown colour, this is due to the enzymatic oxidation of phenolic compounds and shows that the enzymes are highly active in the juice. The rapid appearance of the brown colour of the juice means faster oxidation has occurred due to air bubbles generated during the blending process. It should be noted that the composition of potato juice is similar to that of the potato tuber.

The first experiment that was conducted was spiking potato juice with solutions of 3-CA prepared in water and methanol for different spiking times (0, 2, 5, 10, 20, 30 and 60 minutes) prior to an overnight soaking extraction with methanol. Figure 6:5 shows the extraction recoveries of 3-CA from potato juice versus spiking time.

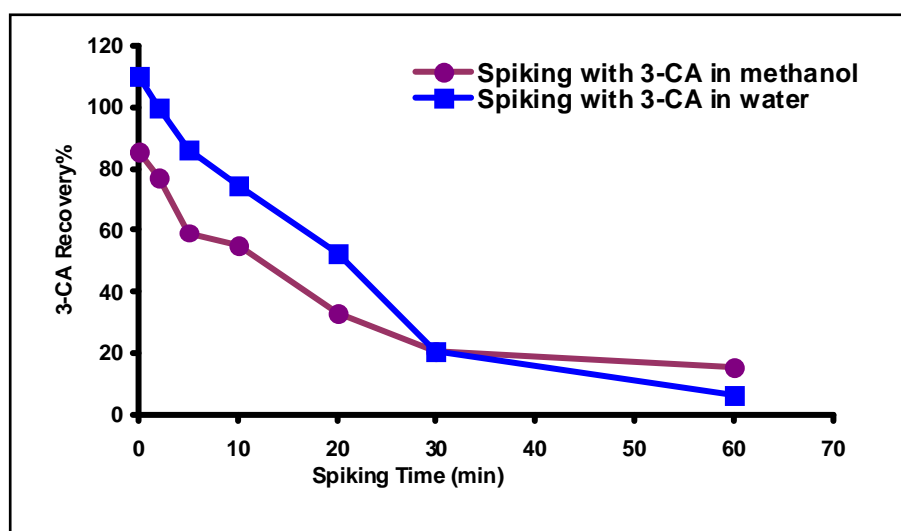


Figure 6.5. The effect of the spiking time on the recovery of 3-CA from spiking potato juice using two solvents of methanol and water.

It can be seen that increasing the spiking time caused a decrease in the extraction recovery after spiking potato juice with 3-CA in both methanol and water. However, spiking with the water solution gave recovery values higher than with methanol, corroborating previous results obtained from spiking peel with 3-CA dissolved in different solvents (see Sections 6.3.2 and 6.3.5). At time zero, the extractant was added to the juice before spiking so there was no direct contact of 3-CA solution with the juice. These results are similar to those in Figure 6:3 when the peel was spiked with 3-CA.

6.3.9.2 Preventing the enzymatic reaction in the potato juice

To assess if this loss of 3-CA was due to enzymatic activity, potato juice was treated by heating to 90 °C to destroy enzymatic activity or adding reducing agents in order to specifically inhibit the oxidase enzymes. Several methods can be used to cause inactivation of the enzymes: addition of chemical additives (i.e. reducing agents, acids and chelating agents), pH alteration and/or temperature (Calder et al., 2011; Pizzocaro et al., 1993; Girelli et al., 2004; Coetzer et al., 2001; Jeong et al., 2005). Heat treatments can be used to cause inactivation of enzymes using temperatures greater than 50 °C (Altunkaya and Goekmen, 2008; Girelli et al., 2004; Chutintrasri and Noomhorm, 2006; Kim, 1995). The addition of antioxidants like ascorbic acid and sodium dithionite can cause the reduction of quinones back to the original phenols and/or remove oxygen from the environment.

In this study, the treated juices were spiked with a methanol solution of 3-CA and left for 1 hour prior to overnight soaking extraction. The recovery results obtained from HPLC analysis of the extract are shown in Table 6:10.

Table 6:10. Recovery values of 3-CA from potato juice treated with different enzymatic inhibitors.

Treatment	3-CA Recovery	RSD% (n = 3)
Sodium dithionite	93	3.8
Ascorbic acid	98	1.1
Heating	99	4.5
No treatment (control)	5	4.8

What is interesting about the data in Table 6:10 is that spiking the treated juices resulted in a high recovery as compared with spiking untreated juice (control). These findings indicate a possible role of oxidase enzymes in the loss of 3-CA. The reason for this loss is not clear but 3-CA may undergo a Schiff base reaction with carbonyl groups produced by the enzymatic oxidation of phenolic compounds. Direct oxidation of 3-CA by enzymatic activity is another possibility that cannot be excluded.

6.3.9.3 Preventing the enzymatic reaction in potato peel

The role of enzymatic degradation of 3-CA in peel was investigated by using ascorbic acid, citric acid, a combination of ascorbic acid and citric acid and sulphuric acid in the spiking solutions. In addition to the antioxidant effect of ascorbic acid, these acids can inactivate polyphenolase enzymes through lowering the pH. The optimum pH for PPO enzyme activity in potatoes is between 4 and 7 depending on the substrate (Lourenco et al., 1992). A combination of ascorbic acid and citric acid has been widely used as an acidifying treatment to prevent the enzymatic browning of potato (Laurila et al., 1998; Langdon, 1987). Citric acid exerts an additional effect by chelation with copper or iron to reduce the enzymatic activity in potato tubers (Singh et al., 1998; Muneta and Kaisaki, 1985; Pizzocaro et al., 1993).

Spiking solutions of 3-CA were prepared with different concentrations of these acids to spike potato peel. Table 6:11 presents the extraction recovery results obtained from the analysis.

Table 6:11. Recovery values for extraction of potato peel spiked with 3-CA solutions containing an enzymatic inhibitors.

Amendment	Concentration	Recovery%	RSD % (n = 5)
Control	-	17	10.7
Ascorbic acid	1%	29	18.4
	5%	45	15.7
	10%	55	2.7
	15%	52	1.3
Citric acid	1%	31	32.4
	5%	46	9.2
	10%	48	6.6
	15%	50	9.8
Ascorbic acid and citric acid	10, 15%	71	9.5
Sulphuric acid	1 M	64	12.6

These results are quite revealing, firstly, using these enzymatic inhibitors greatly improves the recovery of 3-CA from the potato peel but not as much as that attained from potato juice (see Table 6:10). Secondly, there is a clear trend of increasing recovery of 3-CA with increasing concentration of acid. This reveals that the inactivation of the PPO enzyme requires a sufficient concentration of these inhibitors (Pizzocaro et al., 1993). Thirdly, the recovery values obtained when using the same concentration of each of ascorbic acid and citric acid were similar, demonstrating no difference between the action of these two acids. Sulphuric acid gave a slightly higher recovery than either ascorbic acid or citric acid. Thus one of the issues that emerges from these findings is the role of acidity on improving the extraction of 3-CA from potato peel rather than the blocking of enzymatic oxidation by a reduction mechanism.

Nevertheless, a question that must be asked is, what is the role of these acids in improving the extraction of 3-CA from potato samples. The mechanism is not clear but it could be enzymatic inhibition caused by a lowering of the pH below that required for enzymatic activity or that the pH is having an effect on the binding of 3-CA, particularly, the acid and alkaline extraction might have a tendency to break the interaction of 3-CA in terms of a Schiff base reaction with specific potato peel components (Harry Duncan, personal communication).

6.3.10 Investigation of pH effect and ion exchange on extraction of 3-CA from the potato peel

Generally, the skin of a potato tuber constitutes the cork periderm which contains suberin and waxes (Serra et al., 2009). The chemical structure of suberin is a complex polymer consisting of a high proportion of phenolic compounds, fatty acids, fatty alcohols and ω -hydroxy acids combined by ester bounds and peroxide bridges (Kolattukudy and Agrawal, 1974; Serra et al., 2009). The waxes are a complex mixture of lipids that consist of a linear aliphatic chain up to 32 carbon atoms in length (Schreiber et al., 2005). Therefore, the potato skin periderm contains both hydrophilic groups ($-\text{OH}$ and $-\text{COOH}$) and lipophilic groups ($-\text{CH}_2-$ and $-\text{CH}_3$). The presence of the free carboxyl groups is responsible for most of the negative charges on the potato skin (Harry Duncan, personal communication). Therefore, potato skin can exhibit ion exchange properties.

One postulation for the poor extraction of 3-CA from potato peel could be strong binding by ion exchange between the negatively charged groups on the potato skin and the NH_3^+ group of the 3-chloro anilinium ion produced from the amino group. To generate a positive charge on the amino group, the pH of the sample matrix should be below its pK_a . But, the low pK_a value of 3-CA (3.52) (SRC, 2011) and the higher pH range of potato skin (5 – 6) do not support the ion exchange speculation. Because the potato pH is 2 units above pK_a value of 3-CA, only approximately 1% of the 3-CA can be protonated.

Ion exchange binding could be countered by pH adding salts, base and acids. In reviewing the literature, there are numerous studies describing the role of hydrolysis at different pH values on the extraction of chlorinated anilines.

Sodium hydroxide has been used to extract 3-chloroaniline from lettuce and soil. After extraction with acetone and acetone combined with water, which extracted CIPC but did not extract 3-CA, the extracted soil and lettuce were heated with 50% (g/v) NaOH in water. 3-CA was extracted after centrifuging and purification with benzene and hydrochloric acid. Recovery results at 0.1 mg/L level were between 75 – 89 and 79 – 92% in soil and lettuce respectively (Rouchaud et al., 1987).

It was observed that using an aqueous solution of sodium carbonate at pH 11 and 14% (w/v) NaCl extracted aniline metabolites of phenylurea herbicides from juice obtained from food samples by SPME. Recoveries of aniline compounds from samples of potato,

carrots and onions at the 0.02 mg/kg spiking level were found to be greater than 79% (Berrada et al., 2004).

Hsu and Bartha (1974) indicated that, especially at low concentration, the bulk of the chloroaniline moiety becomes tightly bound to the soil and cannot be extracted using salt solutions or by organic solvents, however, alkaline or acid hydrolysis was found to release some, but not all of this bound chloroaniline.

This part of the work involved treatments using acids, bases and neutral salts to examine the extractability of 3-CA. These materials were suggested due to their presence in solution having a considerable effect on changing the pH in addition to their high solubility in the extracting solvent methanol.

Preliminary analysis of the potato peel extract using acids, bases and salts showed recovery results of 3-CA as presented in Table 6:12.

Table 6:12. Recovery of 3-CA using different materials with the extracting solution.

Material	Conc. (M)	Extractant	pH	Recovery %	RSD% (n = 5)
Control	-	CH ₃ OH	ND	11	12.1
NaOH	0.25	H ₂ O	13.4	23	9.6
NH ₃	0.3	CH ₃ OH	11.3	9	5.5
Na ₂ CO ₃	1	H ₂ O	11.8	6	7.7
CH ₃ COOLi	0.4	CH ₃ OH	9.6	12	12.1
CH ₃ COONH ₄	0.3	CH ₃ OH	7.8	6	16.4
CH ₃ COOH	0.1	CH ₃ OH	3.4	17	12.6
CH ₃ COOH	0.9	CH ₃ OH	2.7	16	15.5
CH ₃ COOH	1.8	CH ₃ OH	2.3	22	8.5
H ₂ SO ₄	1	CH ₃ OH	< 1	45	14.5

The results from this table point to low recovery values using basic extractants and neutral salts. However, there is a slight trend of recovery increasing with an increase in the acidity, particularly with sulphuric acid which gave the highest recovery. A further study with more focus on the role of acid in extraction is therefore suggested.

6.3.11 Influence of acidity on the extraction of 3-CA

6.3.11.1 Influence of acidity on chromatographic separation

pH is an important factor in the HPLC separation of ionised compounds. Using high and low pH without control can cause many chromatographic problems like damaging the HPLC column, drifting and poor retention reproducibility for eluting peaks and peak shape deterioration. In addition, too large an injection of a solvent at a different pH to the mobile phase can cause peak shape problems and retention problems. Reducing the injection volume may alleviate this problem. (John Dolan, personal communication).

Usually the typical range for pH stability of normal silica-based C₁₈ columns specified by the manufacturer is from 2 to 8, however the greatest stability of the bonded phase on the column is between pH 3 to 5 at low temperatures. Therefore, injecting a sample of low pH can cause hydrolysis of the bonded phase on the HPLC column.

In order to optimise the separation and quantification determination of 3-CA using an acidic solution, the effect of the acidity on the chromatographic separation was investigated. It was noticed during HPLC analysis that the acidity of acetic acid caused shifting of the peaks of 3-CA and of the internal standard of IPC. The chromatograms showed inconsistency and drifting of the retention times of both peaks from one injection to another, particularly with increasing acid percentage in methanol as shown in Figure 6:6.

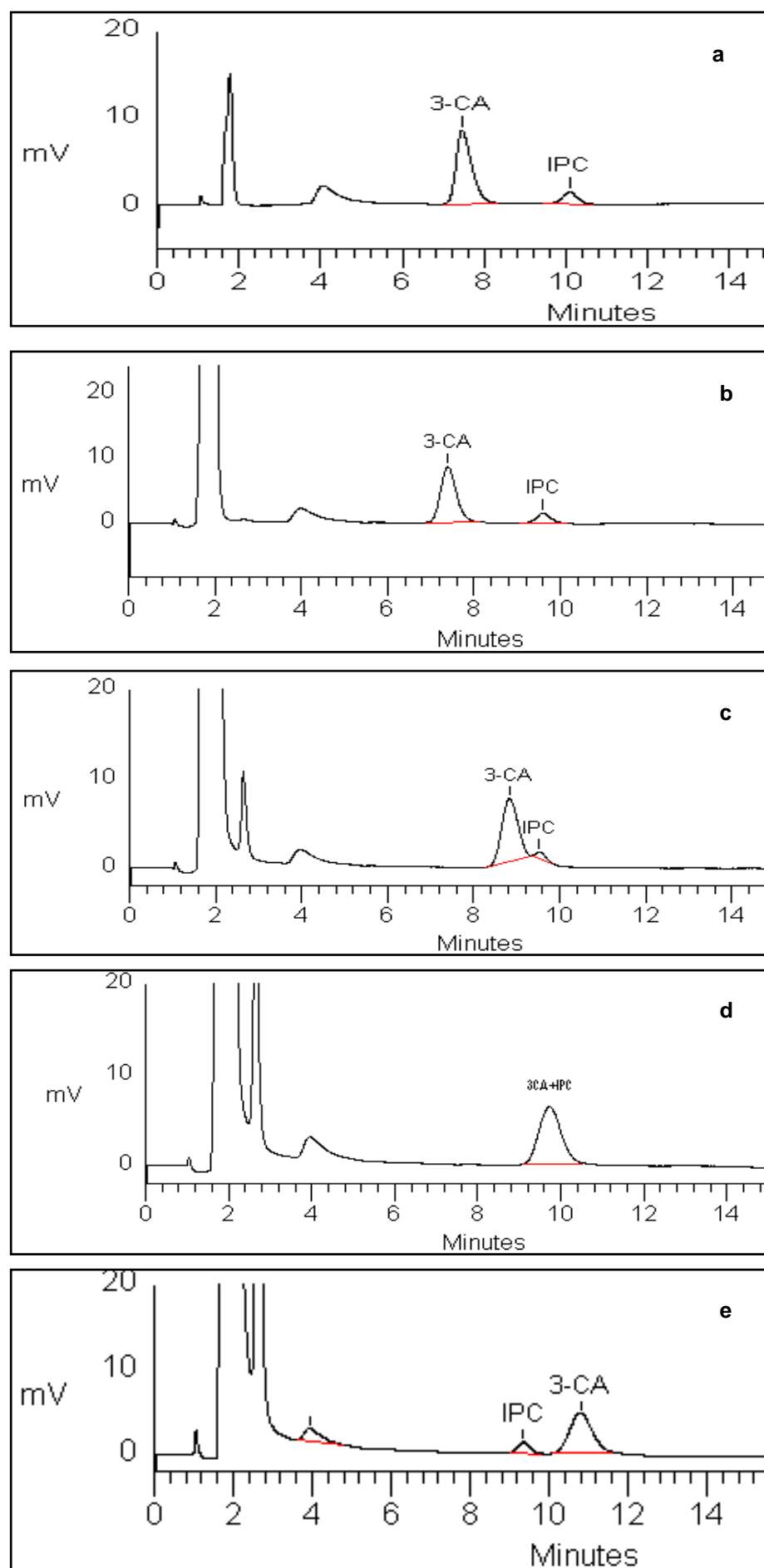


Figure 6:6. The chromatograms of a standard of 1 $\mu\text{g/mL}$ 3-CA and IPC in methanol with different percentages of acetic acid: a- 0%, b- 0.5 %, c- 2.5%, d- 5% and e- 10%.

There are several possible reasons causing variation in the retention time of the analysed compounds. The most common reason is due to the difference between the pH of the sample and the pH of the mobile phase, particularly when the sample contains ionisable species which are known to be inconsistent in their run behaviour in a non-buffered mobile phase (Dolan, 2004). Adjusting the eluent pH is one of the most powerful ways to move peaks around relative to each other if one or more are ionisable (John Dolan, personal communication). The pH of the mobile phase containing organic solvent, water and buffer is assumed to be the same as that of the aqueous fraction (Roses et al., 1996). In addition to these factors, there are other factors which may affect the retention of an ionic species, such as ion pairing with other ions, effects of the ionic strength and co-ion exclusion resulting from ionisation of the residual silanol groups on the silica column (Roses et al., 1996; Lu et al., 2010). Drifting in the retention time of the peak can also result in the case of incomplete equilibration of the column caused by ion-pairing of the mobile phase. Moreover, the presence of carboxylic acid groups in compounds is more sensitive to pH, for example, acetate in acetic acid has some ion-pairing capability because it is more ionised (John Dolan, personal communication).

For ionic compounds, it is not a good idea to run a mobile phase without some pH control. For this reason, starting with a low-pH mobile phase is usually the first choice (John Dolan, personal communication). The concentration of the buffer for HPLC depends on the nature of the sample and the packing material of the column. However, at high concentration of the organic solvent in the mobile phase, buffer solution should not be used. In this present study, buffer solution was not employed due to the high concentration of methanol in the mobile phase and to avoid any damage of the column caused by precipitating salts from the buffer solution onto the column. Additionally, these precipitated salts can damage the pump.

As an alternative and to maintain the efficiency and stability of the column, the pH of the extract sample was adjusted to be between 2 and 8 using NaOH. Additionally, to avoid any salt contamination on the column, rinsing was undertaken using 100% methanol for about 15 – 30 minutes at the end of each day's run. Re-equilibrium of the system with the standard mobile phase (55 – 62% methanol) for at least 30 minutes was performed before at the beginning of a daily analysis in order to return the stability of separation quality on the column.

Figure 6:7 provides representative chromatograms of HPLC analysis of 3-CA in an extracting solution of 1 M H_2SO_4 in 50% methanol containing IPC as the internal standard after adjusting the pH.

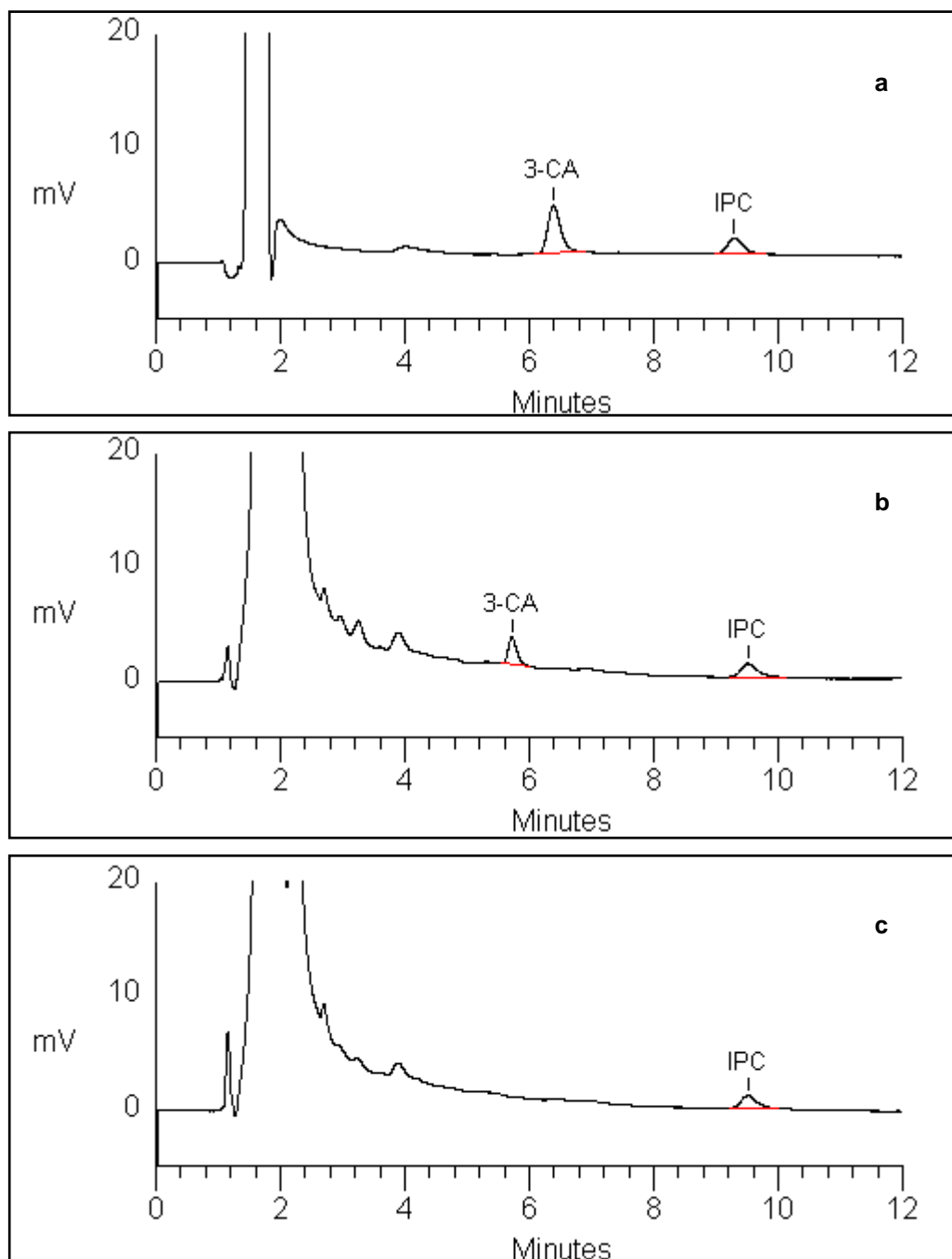


Figure 6:7. Chromatograms obtained using an extracting solution of 1 M H_2SO_4 in 50% methanol at ambient temperature after adjusting the pH in: a- standard of 1 $\mu\text{g/mL}$ 3-CA and IPC, b- extract of spiked potato peel and c- extract of nonspiked potato peel.

As shown from the representative chromatograms, good separation was achieved for 3-CA and the internal standard (IPC) in the sample in acidic methanol after pH adjustment with 1 M NaOH. A slight shifting in retention time was seen for 3-CA, but only during injection of the first few samples, after which the retention time stabilised.

6.3.11.2 Extraction of 3-CA using sulphuric acid in different percentages of methanol

In this study, the high acidity of the methanol promoted the extraction efficiency more than using methanol alone. To investigate the effect of the acidity in the presence of the organic solvent methanol, further work was performed using 1 M sulphuric acid made up in different concentrations of methanol (0, 10, 25, 50, 75, 90 and 100%) to determine what concentration of methanol provided the best extraction for 3-CA. The main purpose of mixing methanol with sulphuric acid is that the organic solvent can wet the surface of the potato peel and penetrate the potato substrates allowing sulphuric acid to break the interaction between the potato peel and the 3-CA. This experiment was conducted using two potato varieties, Nicola and Maris Peer. After overnight extraction, the pH of the extract was adjusted with 1 M NaOH prior to analysis. Recovery results can be seen in Figure 6:8.

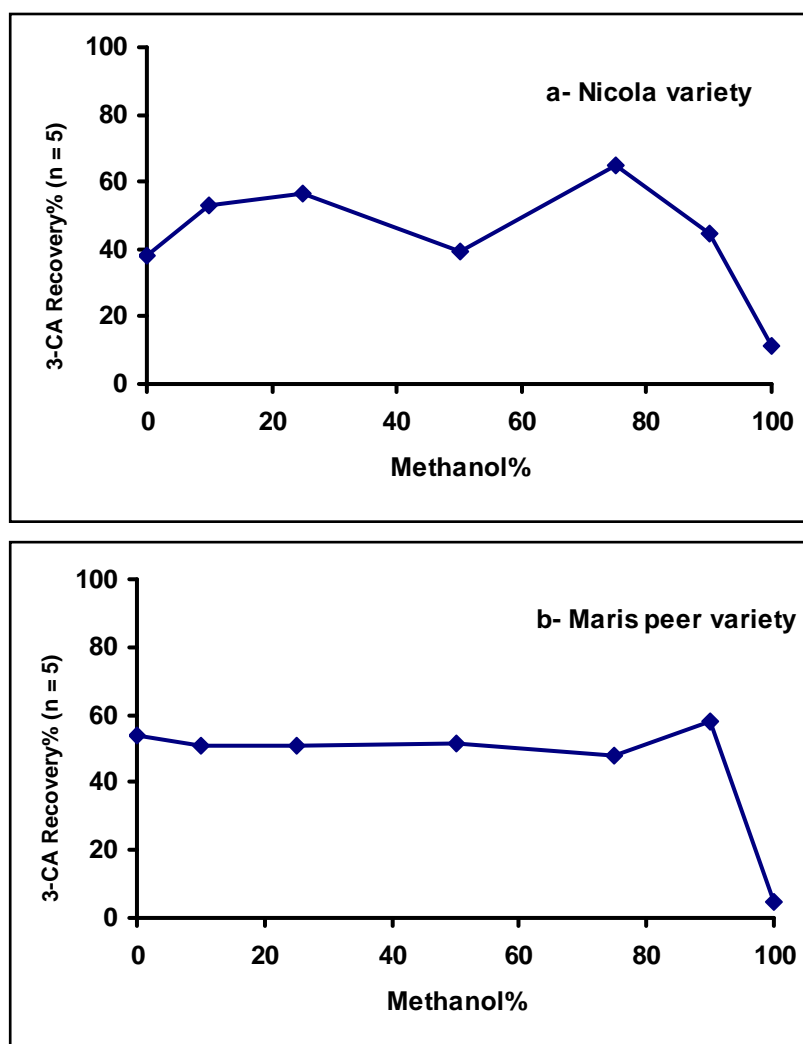


Figure 6:8. The recovery of 3-CA from spiking two potato peel varieties using extracting solution of 1 M H_2SO_4 in different percentages of methanol at ambient temperature.

Although, there is some variability in the recovery values at different percentages of methanol particularly with the Nicola variety it seems that the strong acidity in the extractant is responsible for improving the extraction from both varieties. The recovery of 3-CA is not affected by increasing the methanol percentage in the extractant. The most striking result to emerge from the data is that both varieties showed recovery values in the range of 40 – 60 % in an acidic solution of methanol at all methanol percentages but poor recovery as expected when using methanol alone. As the objective is to extract both residues of 3-CA and CIPC in a potato sample extract, a high concentration of methanol is required to extract the CIPC. Therefore and from an economic point of view, 50% methanol was chosen. This percentage of methanol will be used for optimising the extraction process and investigation other parameters of temperature and extraction time.

6.3.11.3 Influence of temperature on the extraction of 3-CA

Extraction temperature is one of the essential factors for optimising the extraction process. Temperature has a significant effect on the extraction process kinetically and thermodynamically (Zhou and Ye, 2008). It affects the mass transfer rates of the analyte from the matrix to the acceptor phase.

An experiment was conducted to investigate the effect of temperature on the extraction efficiency of 3-CA from spiked peel using an extracting solution of 1 M sulphuric acid in 50% methanol. The investigation involved using two spiking solutions of 3-CA prepared in methanol and water. After overnight extraction at different temperatures (ambient, 22, 50 and 70 °C), the pH of the different extract samples was adjusted by adding 1 M NaOH prior to analysis. Extraction temperature data for 3-CA is shown in Figure 6:9.

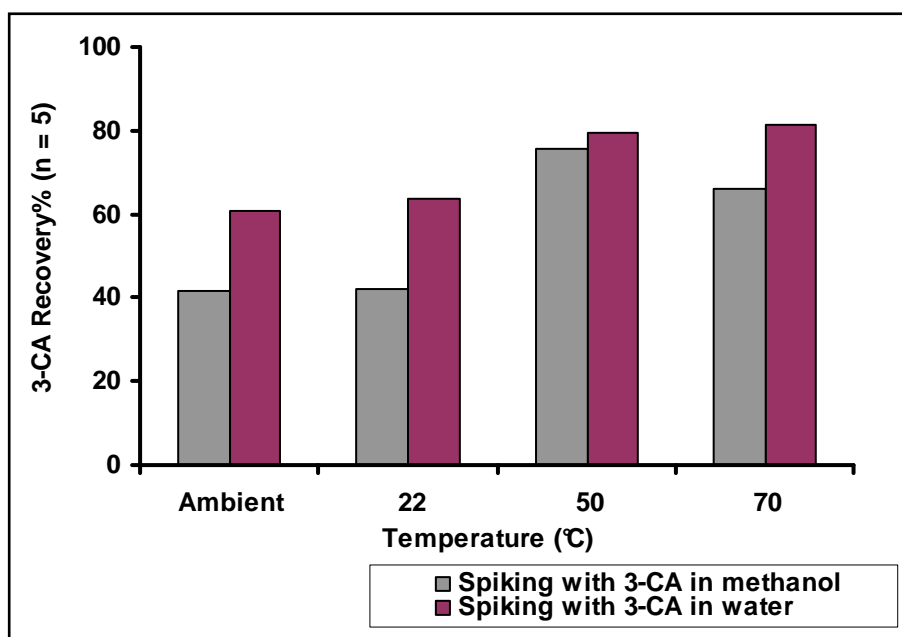


Figure 6:9. The effect of temperature on the recovery of 3-CA from potato peel spiked with two solutions and extracted with a solution of 1 M H₂SO₄ in 50% methanol.

It is apparent from Figure 6:9 that the extraction recovery increased with increasing temperature for both spiking solutions used. The increase in temperature accelerates the diffusion rate and increases the solubility of the extracted substance in the extract increasing the extraction efficiency (Jokic et al., 2010; Cacace and Mazza, 2003). Due to the viscosity and the surface tension of the solvent, the interaction between the target compound and sample matrices can also be disrupted at high temperature (Buldini et al., 2002; Morales-Munoz et al., 2003). Therefore, the high temperature might decrease the binding strength of 3-CA with the potato peel and subsequently increase the distribution rate of 3-CA into the extractant thus increasing the recovery. The figure also shows that spiking with water solution of 3-CA presented recoveries slightly higher than using methanol solution, this seems to be consistent with earlier observations discussed in this chapter (see Sections 6.3.2, 6.3.5 and 6.3.9.1). The best recoveries were obtained at 50 °C and 70 °C and were in the range 66 – 82 % for both solutions of 3-CA used to spike the peel. As there was a little difference in the extraction efficiency between 50 °C and 70 °C, 50 °C was selected for further work.

6.3.11.4 Influence of extraction time on the extraction of 3-CA

The extraction time is another essential factor to be optimised in an extraction procedure. Mostly, the extraction recovery of analytes increases with increasing extraction time until reaching an equilibrium, because the longer time allows more contact between the extracting solvent and sample matrices. However, it is not always practical to use an

extraction time that is long enough for equilibrium to be achieved (Zhou and Ye, 2008). The time required for the analysis is very important when analysing a large number of environmental samples on a daily basis.

To establish the optimal conditions for the extraction procedure of 3-CA using an extracting solution of 1 M H_2SO_4 in 50% methanol, the extraction time factor was investigated. After spiking chopped peel with a methanol solution of 3-CA for 1 hour, the extraction of replicate spiked samples ($n = 3$) was performed over a ranged of different extraction times (2, 6, 12, 18 and 24 hours), all performed in the incubator at 50 °C. After pH adjustment of the acidic extract, the replicates were analysed. As can be seen from Figure 6:10, the extraction recovery of 3-CA increased with extraction time.

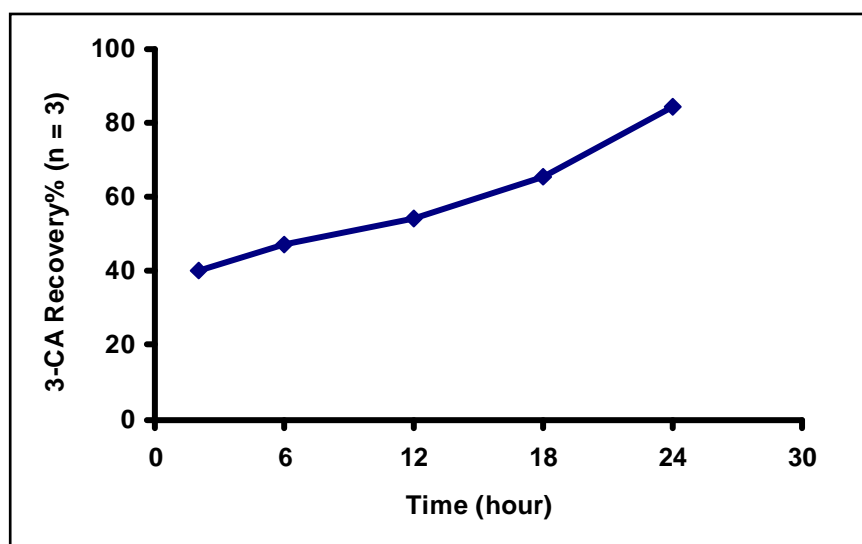


Figure 6:10. Effect of the extraction time on the extraction efficiency of 3-CA using the extracting solution of 1 M H_2SO_4 in 50% methanol at 50 °C.

Even though the extraction did not reach equilibration at the longest time of 24 hours, the best extraction was achieved at 24 hours extraction time where the recovery was found to be 84 % with an RSD% 15.1 for three replicates. However, a higher recovery value may be obtained if an extraction time of greater than 24 hours is used. An extraction time of 24 hours is considered a reasonable and an acceptable time that can be selected for extraction of 3-CA.

6.3.11.5 Influence of acidity on the degradation of CIPC

CIPC is a compound belonging to the well known N-phenyl carbamate group which is solvent and temperature labile, meaning that CIPC is rapidly degraded under improper

solvent and excessive heating (Przybylski and Bonnet, 2009). Additionally, acidifying active solvents like methanol could encourage the hydrolysis and accelerate the degradation process of CIPC initiated by heating. Acidic hydrolysis using dilute (1:1) sulphuric acid combined with heat to boil gently under reflux conditions for 1.5 hours was used to convert the CIPC, to 3-CA and isopropyl alcohol, in both a potato extract sample and milk produced by dairy cows (Gard, 1959; Gard and Ferguson, 1963).

Prior to commencing testing the new method on commercial potato samples, a question needs to be asked as to whether 3-CA can be formed due to the hydrolysis of CIPC in treated potato extracts during extraction, by heating the mixture of sulphuric acid and methanol. CIPC can be hydrolysed under acidic or alkaline conditions, releasing 3-CA (Hajslova and Davidek, 1985; Kearney and Kaufman, 1965; Gutenmann and Lisk, 1964; Romagnol and Bailey, 1966). To investigate this, a solution of 10 µg/mL CIPC was prepared in an extracting solution of 1 M sulphuric acid in 50% methanol (containing 10 µg/mL IPC) and heated under the same conditions as used for the extraction of 3-CA. Comparison of this solution with a standard solution of three compounds (3-CA, IPC and CIPC) prepared at the same concentration in 1 M sulphuric acid in 50% methanol, at ambient temperature, showed no formation of 3-CA as shown in Figure 6:11.

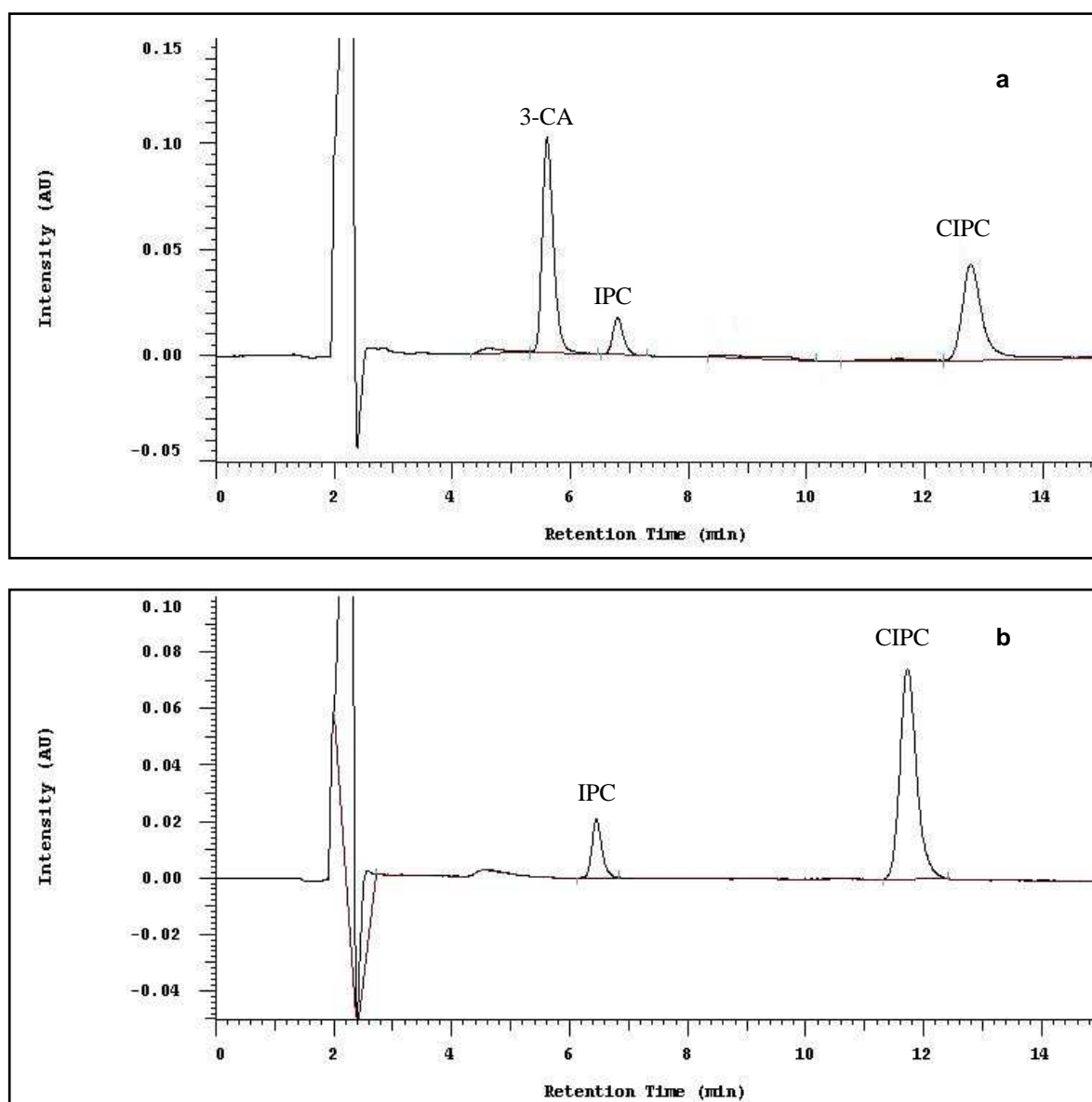


Figure 6:11. Chromatograms of the analysis of a 10 µg/mL standard solution of CIPC prepared in 1 M sulphuric acid in 50% MeOH containing IPC analysed by HPLC-DAD: a- standard of three compounds, no heat treatment and b- heated to 50 °C

6.3.12 Application of the proposed method for the determination of the residues of 3-CA and CIPC in stored potato tubers treated with CIPC

To check the extraction method using a mixture of 1 M H_2SO_4 in 50% methanol at 50 °C for 24 hours, 20 potatoes tubers treated with CIPC from a commercial store, were analysed to determine the residues of CIPC and 3-CA by this new method (see Section 6.3.14). In addition, comparisons were made with methanol (existing CIPC method as summarised in Section 5.3.5.2) and 1 M H_2SO_4 in 50% methanol at ambient temperature. All three extracting solutions contained 10 $\mu\text{g/mL}$ IPC as the internal standard. The analysis of the three extracts was initially performed using the same system (autosampler-SpectraSERIES UV100 HPLC) as described in Section 2.1.2. Chromatograms of the extract showed good separation with high resolution for all three peaks of 3-CA, IPC and CIPC as shown in Figure 6:12.

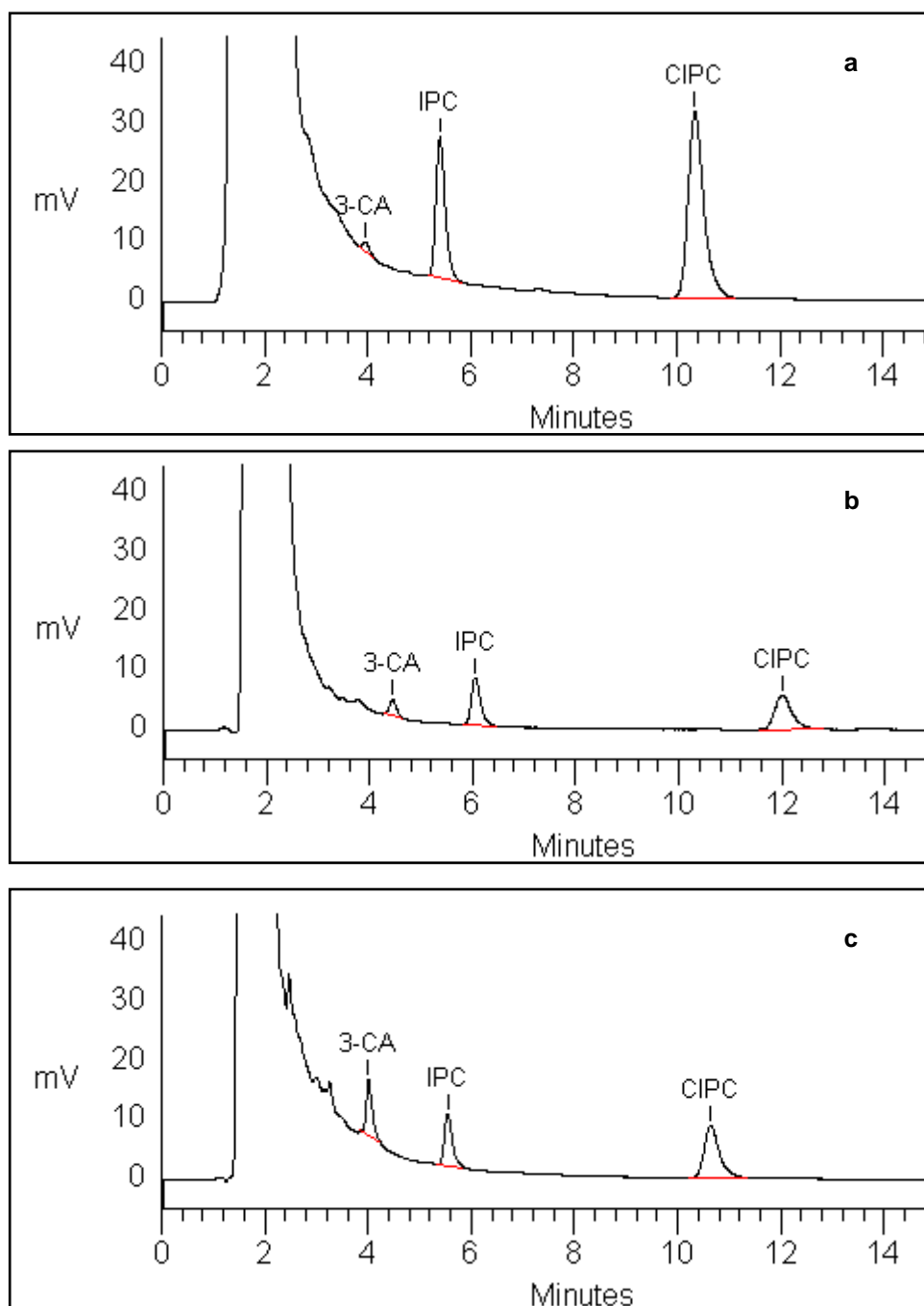


Figure 6:12. SpectraSERIES UV100 HPLC chromatograms of the extract of same potato tuber using different extractants: a- MeOH at ambient temperature, b- 1 M H₂SO₄ in 50% MeOH at ambient temperature and c- 1 M H₂SO₄ in 50% MeOH at 50 °C. (Note: the peak heights in b and c are reduced due to dilution after pH adjustment).

A high peak for 3-CA was noted pointing to a high residue level which was unanticipated. Thus, to confirm the identity of this peak, the analysis of the extract samples was also carried out using the Hitachi DAD HPLC (see Section 3.2.2.2). This system was used to check the purity and identity of the peaks using their spectrum. DAD offers greater ability to analyse peak purity with absorbance measured as a function of retention time and wavelength (Wiberg et al., 2004). Spectra are obtained from the centre, left and the right sides of the peak, the two side spectra are used to calculate peak purity. To confirm the

purity of the peak, the spectrum is compared to the standard. Comparison of these spectra against each other should be close to 100%. Figure 6:13 shows the chromatograms obtained from the analysis of the extract using the DAD-HPLC system.

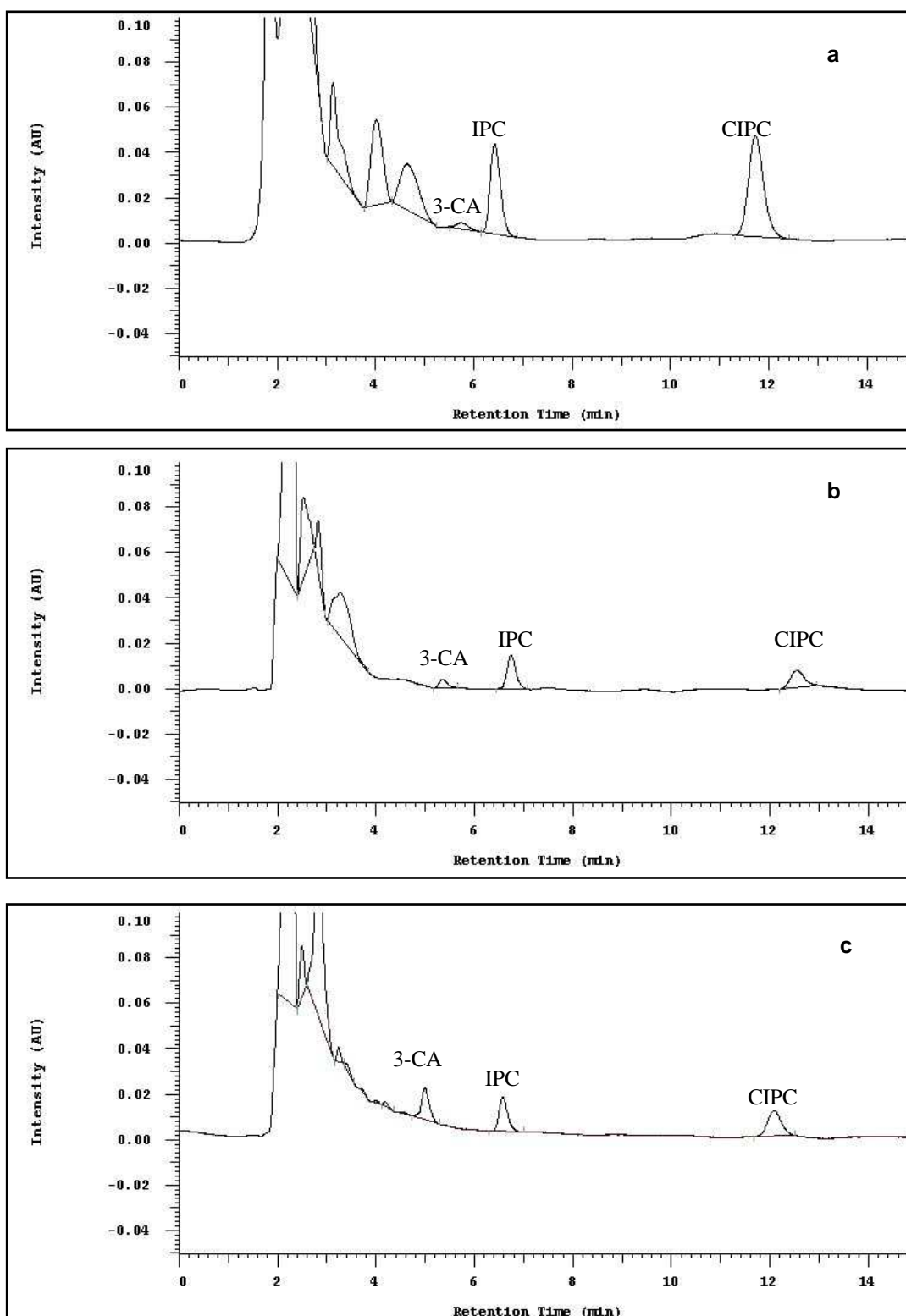


Figure 6:13. DAD-HPLC chromatograms of the extract of the same potato tuber using different extractants: a- MeOH at ambient temperature, b- 1 M H_2SO_4 in 50% MeOH at ambient temperature and c- 1 M H_2SO_4 in 50% MeOH at 50 °C. (Note: the peak heights in b and c are reduced due to dilution after pH adjustment).

A good separation of 3-CA, CIPC and the internal standard IPC with good resolution was obtained using DAD-HPLC system at 65% methanol as the mobile phase, flow rate 1 min/mL and the same Phenomenex[®] column (ODS-2 250 mm x 4.6 mm 5 μ m Spheredclone) coupled with guard column. The DAD-HPLC system confirmed the identity of the three peaks and shows peak purity greater than 95%. A further test was made by adding a solution of 10 μ g/mL of 3-CA to the extract of some potato tubers. The chromatogram showed an increase in the peak area of 3-CA, no peak splitting and the purity of the peak was more than 95%, thus confirming the identity of the 3-CA peak.

To assess any difference between the two HPLC analyses, comparisons were made by plotting correlation graphs for the residues results of 3-CA and CIPC as shown in Figures 6:14 and 6:15.

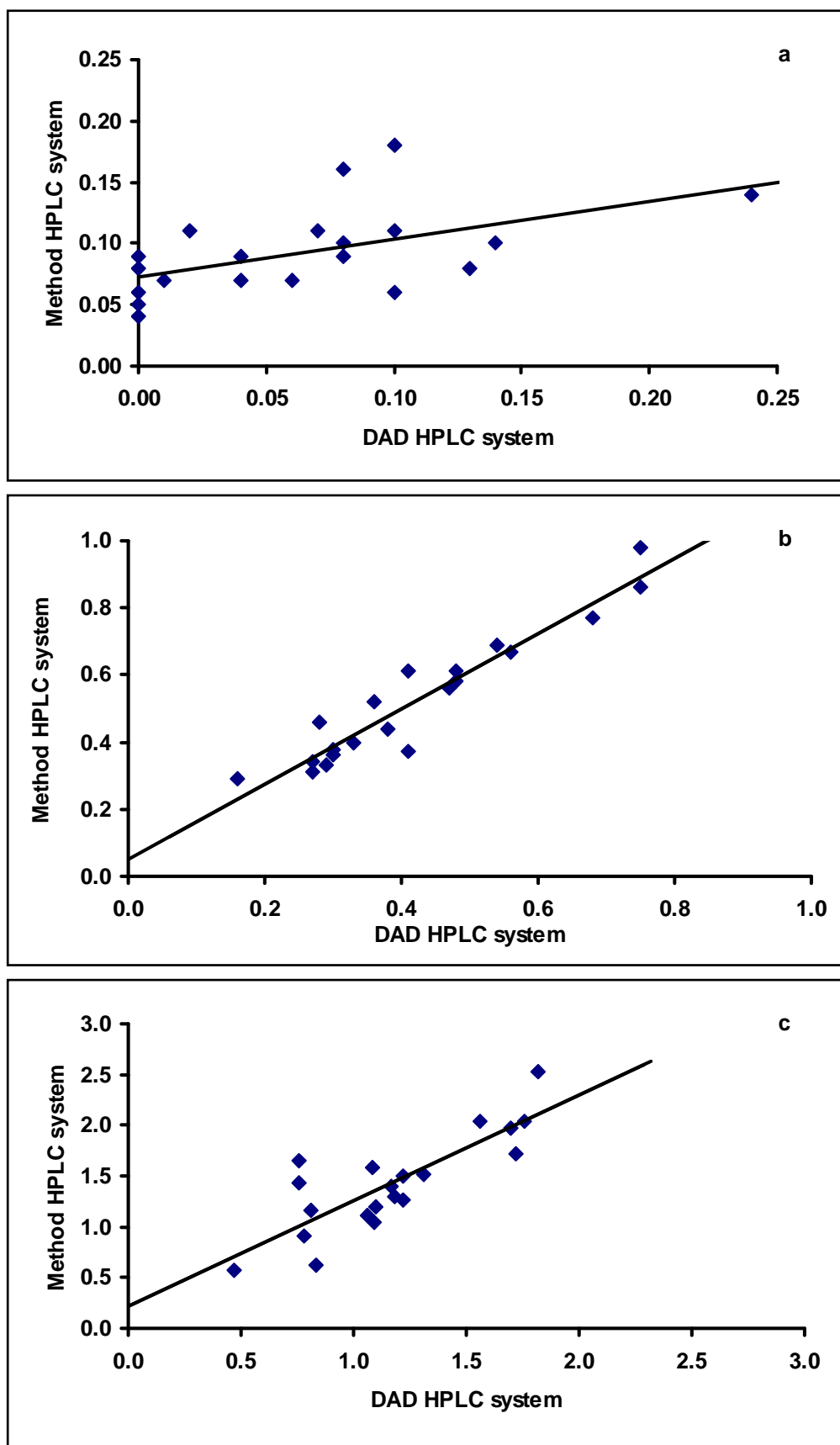


Figure 6:14. Correlation between the residue of 3-CA analysed by two HPLC systems and extracted by: a- MeOH at ambient temperature, b- 1 M sulphuric acid in 50% MeOH at ambient temperature and c- 1 M sulphuric acid in 50% MeOH at 50 °C.

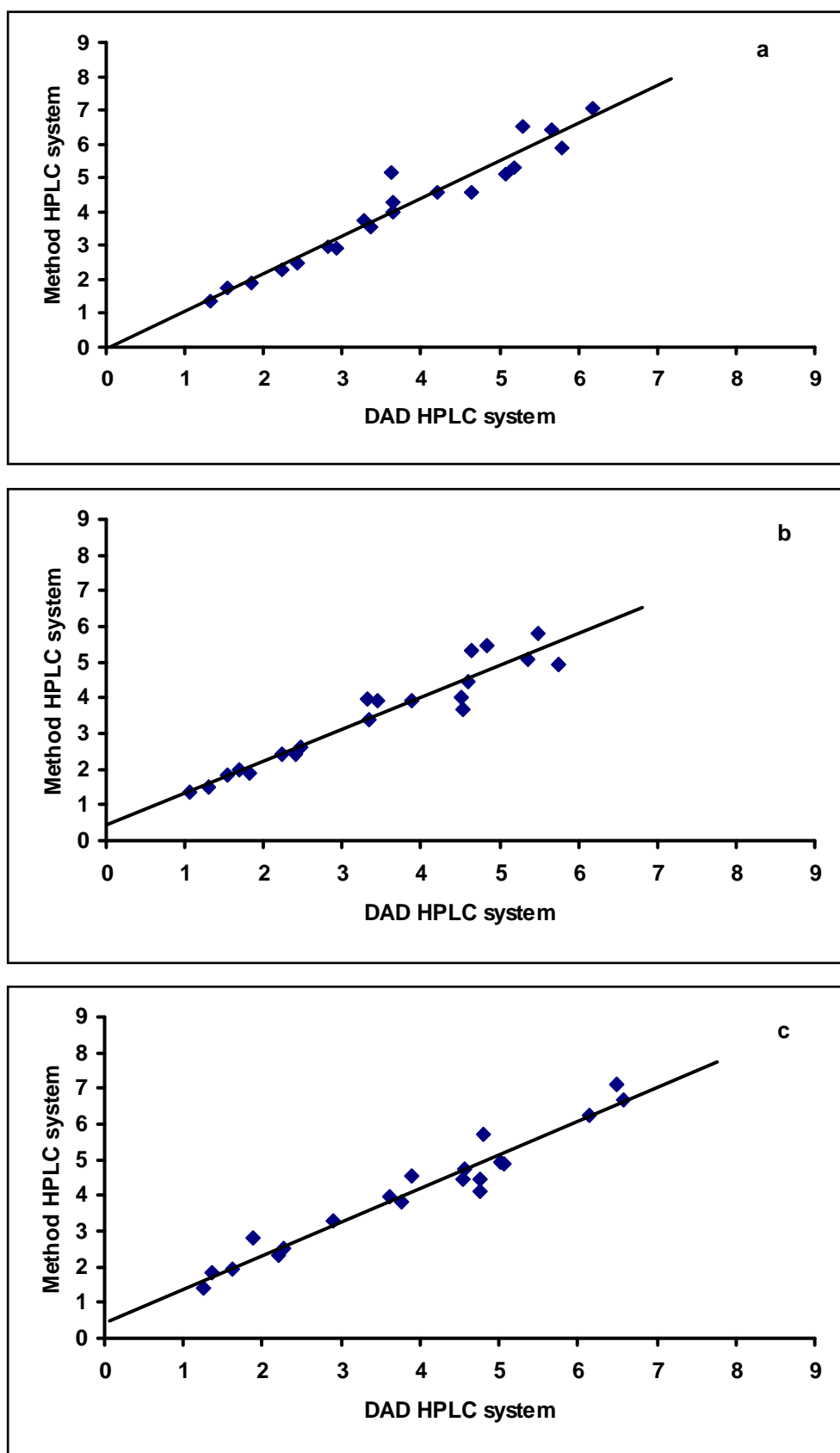


Figure 6:15. Correlation between the residues of CIPC analysed by two HPLC systems and extracted by: a- MeOH at ambient temperature, b- 1 M sulphuric acid in 50% MeOH at ambient temperature and c- 1 M sulphuric acid in 50% MeOH at 50 °C.

Good correlation can be seen from these figures between the two HPLC systems. The SpectraSERIES UV100 HPLC system gave slightly higher values than the DAD-HPLC system. However, although statistical analysis using a paired t-test (Table 6:13) showed significant differences ($p < 0.05$) for both 3-CA and CIPC, in analytical practice the differences have no significant effect since the mean differences of the residues are very small.

Table 6:13. Paired t-test of two HPLC analyses of 3-CA and CIPC residues after extraction of 20 potato tubers.

Extractant	Mean difference		p- value	
	(n = 20) mg/kg			
	3-CA	CIPC	3-CA	CIPC
MeOH at ambient Temp.	0.03	0.36	< 0.05	< 0.05
1 M H ₂ SO ₄ in 50% MeOH at ambient Temp.	0.10	0.08	< 0.05	< 0.05
1 M H ₂ SO ₄ in 50% MeOH at 50 °C	0.26	0.21	< 0.05	< 0.05

The results obtained from the analysis of the residue values of 3-CA and CIPC in the 20 potato tubers using three extraction methods are shown in Figure 6:16 and 6:17.

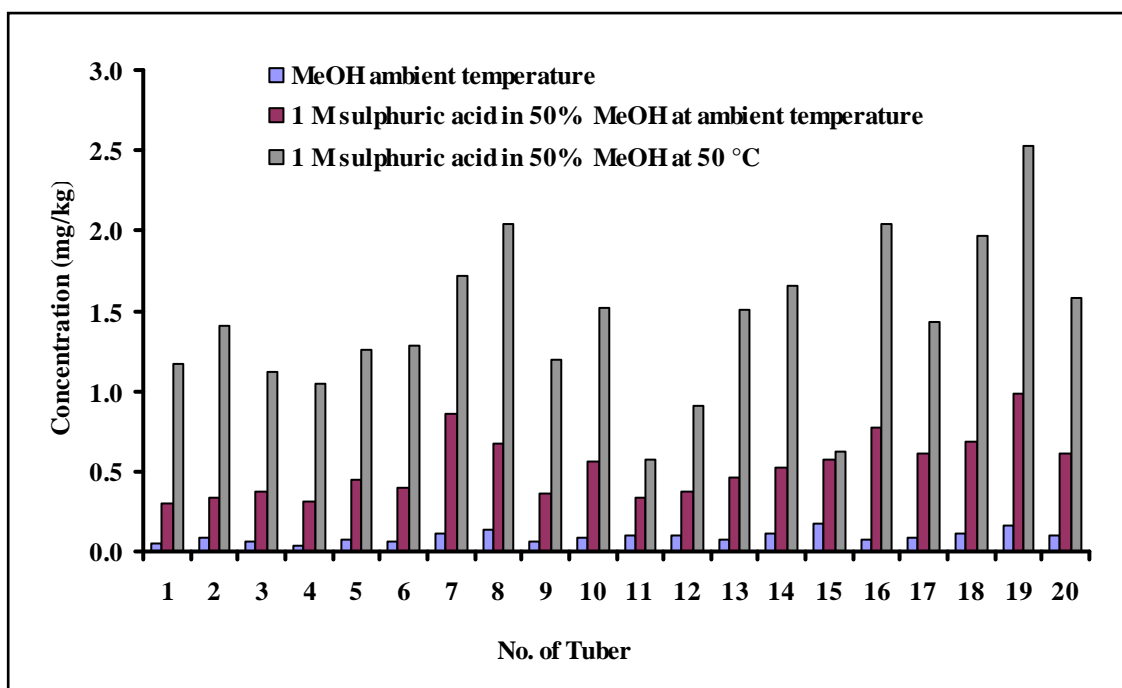


Figure 6:16. 3-CA residue in 20 potato tubers treated with CIPC and extracted by three different methods and analysed by HPLC (SpectraSERIES UV100).

The histogram in Figure 6:16 indicates that there is a clear trend of increase in the residue concentration of 3-CA using the three extraction methods, with the proposed method of using 1 M H_2SO_4 in 50% MeOH at 50 °C extracting significantly more than either that extracted at ambient temperature or that extracted using methanol alone (mean residue values were 1.43, 0.53 and 0.09 mg/kg respectively). These results demonstrate that the new extraction method (1 M H_2SO_4 in 50% MeOH for 24 hours at 50 °C) shows the same pattern of relative recovery of 3-CA in commercial potato samples treated with CIPC as was obtained for the spiked samples.

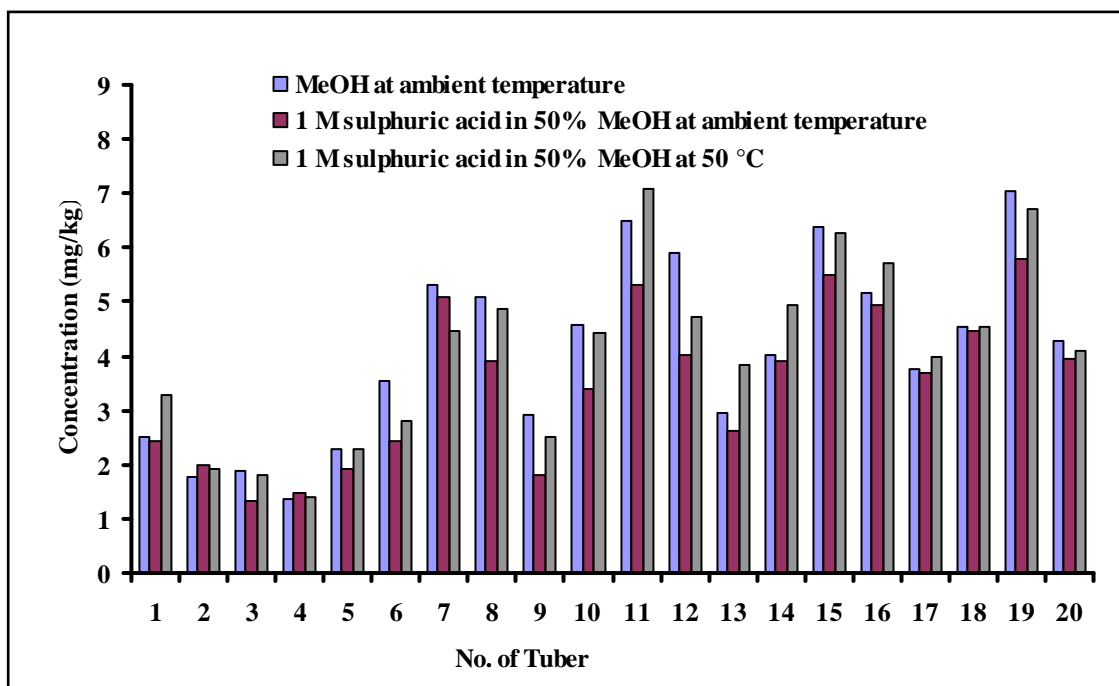


Figure 6:17. The residue of CIPC in 20 potato tubers treated with CIPC and extracted by three extraction methods and analysed by HPLC (SpectraSERIES UV100).

The mean CIPC residue values determined for these 20 potato tubers using the three extraction methods (methanol, 1 M H_2SO_4 in 50% MeOH at ambient temperature and 1 M H_2SO_4 in 50% MeOH at 50 °C) were 4.09, 3.50 and 4.09 mg/kg respectively. Nevertheless, a question that must be asked is, does the new method give the same measurement for the extraction of CIPC from treated potato samples compared with the method developed in Chapter 5 using the methanol solution alone. To investigate this, comparisons between the two methods were made and the residue data were plotted as is shown in Figure 6:18.

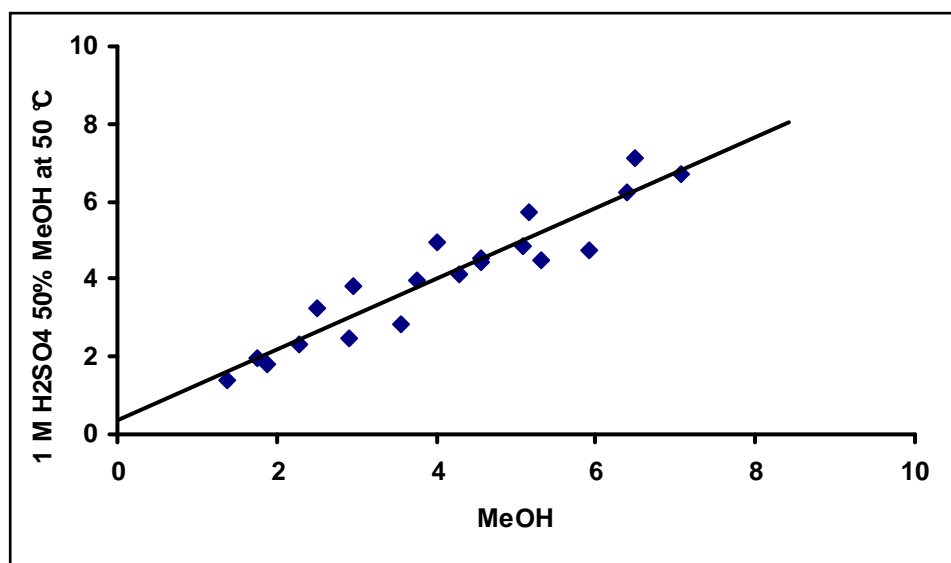


Figure 6:18. Correlation between the residue of CIPC extracted by the standard method using MeOH and the new method using 1 M H₂SO₄ in 50% MeOH at 50 °C and analysed by HPLC system (SpectraSERIES UV100).

It is apparent from this figure that there is a good correlation between the two extraction methods. Further statistical analysis using a paired t-test showed that there was no significant difference ($p > 0.05$) between the two methods used to extract the residue of CIPC in 20 treated tubers. The conclusion that can be drawn is that the new method is suitable for extraction of the residue of CIPC as well as that of 3-CA.

Table 6:14 shows the residues of both of 3-CA and CIPC in these 20 tubers using 1 M H₂SO₄ in 50% MeOH at 50 °C.

Table 6:14. The residues of 3-CA and CIPC in 20 potato tubers treated with CIPC.

Residue of 20 tubers (mg/kg)	3-CA	CIPC
Minimum	0.57	1.41
Maximum	2.53	7.09
Mean	1.43	4.09

It can be seen from the data in the table that the most striking result is that a high residue concentration of 3-CA was detected in these potato samples whereas CIPC residues were lower than maximum residue level (MRL) of 10 mg/kg.

It seems possible that this residue of 3-CA is due to degradation of CIPC during application in the store (Park et al., 2009; Nagayama and Kikugawa, 1992; Worobey and Sun, 1987; Worobey et al., 1987; Park, 2004). CIPC was applied to the potato tubers as solid formulation, melting at 37 °C and fogged at 450 °C through metal pipes. Degradation of CIPC might occur due to pyrolysis on contact with metal surfaces at high temperatures resulting in the formation of 3-CA (Park et al., 2009; Romagnol and Bailey, 1966). These potatoes were analysed at the end of the season, meaning that they may have received several applications of CIPC. Another possible reason for this residue of 3-CA is that microbial degradation of CIPC residue might have occurred during the long storage period (Kleinkopf et al., 1997; Kaufman and Kearney, 1965; Wolfe et al., 1978; Rouchaud et al., 1986a). Furthermore, 3-CA is used to synthesise CIPC commercially by reacting with isopropylchloroformate so it may be present in the CIPC formulation as a contamination, but only at very small levels (0.05% of CIPC weight) (Worobey and Sun, 1987; Park et al., 2009).

6.3.13 Effect of fogging temperature and the number of CIPC applications on the residue levels of 3-CA and CIPC in stored potatoes

This work focussed on the effect of fogging temperature application on the residue of 3-CA and CIPC in potato tubers under commercial store conditions at the start of the storage season 2010 – 2011. Comparison was made between high temperature fogging (450 °C) applied to a bulk store at 14 g/tonne, with low temperature (270 °C) application of a box store at a rate of 12 g/tonne, under commercial conditions (9 °C). Further samples were obtained following a second application at low temperature (270 °C) in the box stores. Potatoes tuber samples were taken from these stores and extracted using the new extraction method of 1 M sulphuric acid in 50% methanol for 24 hours at 50 °C. The results obtained from the analysis are shown in Table 6:15.

Table 6:15. Residue levels of 3-CA and CIPC in commercially treated potatoes in UK stores for season 2010 – 2011 under different applications.

No. of tuber	3-CA residue (mg/kg)		CIPC residue (mg/kg)			
	270 °C		450 °C		270 °C	
	1 st	2 nd	1 st	1 st	2 nd	1 st
1	0.05	0.06	0.09	3.52	8.02	0.40
2	0.10	0.33	0.05	1.15	3.41	0.38
3	0.05	0.44	0.12	1.36	4.03	0.44
4	0.12	0.19	0.15	2.22	1.32	1.29
5	0.13	0.26	0.10	1.95	3.48	0.56
6	0.08	0.40	0.13	2.36	2.37	0.63
7	0.09	0.50	0.09	2.03	6.38	0.48
8	0.12	0.24	0.05	1.72	4.23	0.48
9	0.07	0.78	0.14	1.89	6.06	0.33
10	0.20	0.37	0.05	1.40	3.17	1.42
Minimum	0.05	0.06	0.05	1.15	1.32	0.33
Maximum	0.20	0.78	0.15	3.52	8.02	1.42
Mean	0.10	0.36	0.10	1.96	4.25	0.64
ANOVA test	a*	b	a	c	d	c

* Different letters refer to a significant difference ($p < 0.05$) Tukey HSD

The Tukey test results showed no significant difference between high and low temperature fogging after a first application of CIPC for both residues of 3-CA and CIPC. A significant increase was found between the first and second application at 270 °C indicating a possible build up during storage as obtained in Table 6:14 which shows high residue levels in the end of storage season samples. The reason for this increase may be due to repeated application and/or microbial degradation of CIPC which cannot be excluded.

Thermal degradation of CIPC during application was investigated by Park et al., (2009) to determine the influence of burner temperature, formulation flow rate and the use of a metal pipe on the formation of 3-CA as a product of CIPC breakdown. It was found that a high burner temperature (600 °C) caused more breakdown of CIPC than a lower temperature (475 °C), whereas no breakdown occurred at 190 °C. 3-CA was found to be present in air samples that were taken from treated stores using a high burner temperature (600 °C) application but none was found in the corresponding air samples at 190 °C. Moreover, it

was found that using a metal pipe resulted in more 3-CA than when either a plastic pipe or no pipe was connected to the fogger machine. These results suggest both direct thermal degradation as a result of the high burner temperature and the catalytic effect of the metal pipe are responsible for the presence of 3-CA in potato stores (Park et al., 2009).

Table 6:15 also showed no significant difference between the residues of CIPC at high and low temperature applications of CIPC whereas a significant increase was found between the first and second application at low temperature (270 °C).

Due to the time limitation this research had to be stopped at this point, however, a detailed study is required to investigate both aspects of temperature and time and in addition, the catalytic effect of the metal pipe used in the CIPC fogger application.

6.3.14 Summary of final extraction method for simultaneous determination of 3-CA and CIPC from potato peel samples

Procedure

The proposed method for the extraction of the potato sprout inhibitor chlorpropham (CIPC) and its metabolite 3-chloroaniline (3-CA) from potato peel samples can be summarised briefly as below:

1. Washing and drying potato tubers.
2. Recording the weight of each tuber.
3. Peeling the potato with a stainless steel peeler and recording the weight of peel.
4. Chopping the peel into fine pieces then mixing to obtain a homogenous sample.
5. Soaking 2.5 g chopped peel sample in 20 mL of an extracting solution containing 1 M H_2SO_4 in 50% methanol and an internal standard of propham (IPC) for a period of 24 hours at 50 °C.
6. Adjusting the pH of the extract sample (2 mL) by adding 1 M NaOH which was made up to 5 mL with methanol then filtering the sample through a 0.2 μm PTFE membrane syringe prior to transfer into an HPLC vial for analysis.

Chromatographic conditions

The chromatographic parameters for this method are summarised as follows:

- Column: Phenomenex[®] (ODS-2 250 mm x 4.60 mm 5 μm Sphereclone)
- Guard column: Phenomenex[®] Security Guard[™]
- Detector: SpectraSERIES UV100
- Wavelength detection: 210 nm

- Mobile phase: 62 % methanol
- Flow rate: 1.5 mL/min
- Chromatographic run: 15 minutes.
- CIPC retention time: ~ 13 minutes.
- IPC retention time: ~ 7 minutes.
- 3-CA retention time: ~ 5 minutes
- Injection volume: 20 μ L
- Column temperature: 25 $^{\circ}$ C

6.4 Conclusion

In reviewing the literature, no suitable published method for the determination of 3-CA in potato peel was found. The work in this chapter demonstrated initially poor extraction of 3-CA from the potato peel using different potato varieties, solvents, extraction methods, treatments and different parts of the potato tuber.

Due to the structural complexity of the potato matrix the formation of bound residues of 3-CA is not well understood. However, the poor extraction of 3-CA was speculated to be caused by four possible mechanisms including: volatilisation, reaction with potato components, enzymatic activity and ion exchange processes related to pH.

Although 3-CA has a high vapour pressure, under the experimental conditions of this study there was no measurable loss of 3-CA by volatilisation.

The Schiff base reaction and hydrogen bonding may play a very important role in the reaction of the amino group of 3-CA to carbonyl and quinone groups, which are abundant in potatoes. However, under the experimental conditions used, no reaction of 3-CA was found to occur with any of the other potato components studied.

The results of this investigation show a possible role of oxidase enzymes in the loss of 3-CA due either to the Schiff base reaction with quinone groups of enzymatic oxidation products of phenolic compounds in potatoes or direct oxidation of 3-CA by enzymatic activity.

Inhibition of enzymatic activity by antioxidants or acidity was shown to enhance the extraction of 3-CA.

The suggested binding mechanism by ion exchange is based on the electrostatic attraction between the charged functional group of the amine group on 3-CA ($-\text{NH}_3^+$) to the negatively charged groups present on the potato peel (which are predominantly as carboxyl groups). Binding of 3-CA to potato peel substrates by ion exchange seems unlikely as the pK_a value of 3-CA is lower than the pH of the potato. Changing of the pH of the extracting solution indicated that neutral and alkaline solutions did not promote the extraction of 3-CA from spiked peel. However, high acidity using sulphuric acid combined with methanol as an extracting solution improved recovery. The extraction process was optimised for temperature and extracting time. Using a mixture of 1 M H_2SO_4 in 50% methanol as an

extracting solution for 24 hours at 50 °C increased the extraction recovery of up to 85%. This procedure represents an efficient and acceptable method for the extraction and analysis of 3-CA from potato peel samples and furthermore it can be used for the simultaneous extraction of CIPC.

The developed method was applied to potato samples from commercial stores to determine the residue of 3-CA and CIPC in potatoes that had been treated with CIPC (as it is an important issue for the potato industry). Additionally, potatoes were taken from different UK stores during the storage season 2010 – 2011 to compare the formation of 3-CA using high and low temperature fogging of CIPC (450 °C and 270 °C), two different application rates (14 and 12 g/tonne respectively) in addition to the first application, a second application at 270 °C.

Chapter 7: General discussion and recommendations

7.1 General discussion

Annually, the UK produces up to six million tonnes of potatoes and approximately four million tonnes of this production is stored for the fresh market and for food processing. The storage period starts from September or early October and it may be last up until the next harvest season, which may in actual fact, be longer than the time that the tubers spent in the planted area. Therefore, it is important that this length of storage is able to maintain potato quality and avoid sprouting, to meet the specific demands of the commercial market and human consumption. The storage requirement for the fresh market is noticeably different to those requirements for the processing market. During storage, potatoes destined for the fresh market are held at a low temperature, usually below 4 °C and in addition may also be treated with CIPC or ethylene to control sprouting or they may be stored at 2 °C without chemical treatment (Cunnington, 2008). Potato tubers for processing purposes are held at temperatures ranging from 8 to 10 °C taking into consideration the potato variety, potato sugar status and storage time. These higher temperatures are required in order to minimise reducing sugar accumulation in potatoes, however, the higher temperature means that sprouting can be expected. Hence, sprout suppressants are essential to prolong the dormancy period of the potato, thus avoiding sprouting for longer. CIPC is used as the main sprout suppressant for the processing market.

Considerable research studies have been made by the UK potato council represented by Sutton Bridge Experimental Unit (SBEU) in collaboration with the University of Glasgow and others to improve the efficiency of sprout control by CIPC application. However, its application at the present time is still the subject of concern due to the presence of its residue and the residues of its degradation products (mainly 3-chloroaniline) on potatoes and in potato wash water. Although CIPC residues are mostly located in the potato peel, which can be removed by peeling, most supermarkets demand products that are free of chemical residues. Searching for alternatives, in particular naturally occurring sprout suppressants, may meet this demand. To date, there is no replacement for CIPC in the UK. However, application of ethylene to potatoes destined for processing and long term storage in high temperature stores is a step forward and is currently under study. There are concerns about ethylene application, regarding the formation of the carcinogen acrylamide

during frying at high temperatures, due to the production of high levels of reducing sugars. In addition, the ethylene used may be synthesised from ethanol, which can result in some residues from this alcohol on the potato tuber. Furthermore, the reaction of two molecules of ethanol may produce ether compounds resulting in potato tubers having a sweet taste (Harry Duncan, personal communication).

Nowadays, globally, attention is being given towards using naturally occurring essential oils as sprout suppressant chemicals, particularly caraway seed oil and spearmint oil which are sources of carvone. These products are highly volatile and are extracted from plants and therefore can be certified to apply for use on organic potatoes. Carvone is available now in Europe but further studies are required to understand its mode of action in the control of sprouting.

1,4-Dimethylnaphthalene can be an acceptable replacement for chlorpropham because it is a naturally occurring compound in potatoes. In addition, its volatility may reduce residues on potato tubers during long term storage. Currently, 1,4-DMN is used commercially in some countries (e.g. USA, Canada and New Zealand) in the world, as the active ingredient in products such as 1,4SIGHT, 1,4SHIP and 1,4SEED. These products are liquid formulations which can be applied to potatoes without the need for a solvent, thereby reducing concern from the risk of the solvent used. Prior to a registration decision for the introduction of 1,4-DMN for commercial use in the UK, more investigation is required to ensure that its use does not cause any source of concern to human health or the environment. Although, in reviewing the literature no information is indicated as to the carcinogenicity or toxicity of 1,4-DMN, high residue levels on the potatoes must still be considered for human health and environmental risks. Therefore, many issues have to be addressed regarding the minimum rate required to control sprouting, particularly as there is very limited information about this issue. It is also important to monitor the residue levels to avoid high residues that may produce an undesirable taste in the potato tubers. Moreover, a detailed risk statement of its toxicity is also essential. All of these considerations should be monitored in the context of the residue levels of this potato sprout inhibitor (1,4-DMN) in stored potatoes and in other environmental samples. Therefore, the analysis of this compound in these samples requires specific regulated methods to be developed and validated.

The first step in an analytical method is to separate the intended compound from the sample matrix using an efficient extraction procedure with a suitable solvent capable of

transferring the analyte from the sample matrix to the solvent solution. This step is followed by cleaning up of the extract, freeing it from any interference and allowing for the final step of quantitative detection. Following method development, validation of the acceptability of the method for application should be proved.

Researchers at the University of Glasgow started investigating 1,4-DMN as a potato sprout inhibitor in 1975, developing reliable analytical methods. To date very little information has been published regarding the analytical methods for this polycyclic aromatic hydrocarbon compound. GC and HPLC are ideal techniques and are commonly used for analysis in the quality control of PAHs in food and environmental samples (Stanciu et al., 2008). However, GC analysis of PAHs is subject to thermal decomposition and adsorption onto the GC inlet and column. In addition, GC in combination with FID detection provides lower sensitivity than with HPLC-UV and in addition it is more likely to be subject to background interferences from the sample matrix (Cai et al., 2009). HPLC in combination with UV or DAD detection offers high sensitivity with high specificity. The HPLC-UV technique is an improvement over the GC method since no derivatisation step is necessary prior to analysis (Kashyap et al., 2005). Therefore, HPLC-UV was preferred and selected for this study for the analysis of 1,4-DMN and subsequently for CIPC and its metabolite 3-CA.

This study started by validating an HPLC separation method for 1,4-DMN using 2-MeN as internal standard. 2-MeN was selected from the different isomers and related compounds due to its structural similarity to 1,4-DMN and its good resolution from 1,4-DMN in a mixed standard solution compared with other isomers. Moreover, the solubility of 2-MeN in water is higher than other related naphthalene compounds, which is important when investigating 1,4-DMN in waste water.

Testing the HPLC chromatographic system is required to ensure system suitability for the target application. System suitability tests are an integral part of chromatographic analysis and should be used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis (Krishna et al., 2010). Suitability of the HPLC system is proved by consistent performance during replicate injections of the standard and high separation efficiency. Three isocratic RP-HPLC systems for the analysis of 1,4-DMN and 2-MeN were tested using the same mobile phase concentration of acetonitrile (70%) and column, to select the best system for continuing this study. The three HPLC systems were used to compare two aspects, including the sample injection method and the detector

sensitivity. Sample injection can be done either manually using a manual injection valve or automatically by an autosampler. In this test, both autosampler and manual injector were used. The choice of the detector is one of the main considerations that should be taken into account when developing an HPLC analytical method which principally depends on the limit of detection required for the target analyte. Two detectors were compared, multi wavelength (Merck Hitachi L-4500 diode array) and single wavelength (SpectraSERIES UV100) detectors. Excellent separation was achieved using the same chromatographic conditions with all three HPLC systems but using an autosampler coupled with a single wavelength detector system gave the most precise results with lower limits of detection and quantification for 1,4-DMN. Autosampler injection is more frequently used in standard HPLC equipment as it provides better reproducibility than manual injection. Therefore, this system was selected for quantification of 1,4-DMN in laboratory and environmental samples and later coupled with column oven and cooling system to overcome temperature fluctuation, to achieve more consistent performance.

However, at the time of undertaking this study, the supply of acetonitrile was severely reduced due to the global economic slowdown of 2008 – 2009, which resulted in a shortage of demand for acrylonitrile products. Acetonitrile is obtained as a byproduct of acrylonitrile manufacture. Another reason for the shortage of ACN is that a major production facility for ACN in the USA on the Gulf Coast was shut down due to damage from Hurricane Ike (Purdie et al., 2009; Gaytan, 2009). This shortage resulted in raising the price of ACN in Europe up to 5 fold and reducing the supply to laboratories by up to 80% (Purdie et al., 2009). Before this shortage, acetonitrile was commonly used for many reasons such as, its high polarity, high solubility of most organic species, relatively low price and high availability, therefore searching for other solvents seemed to be unnecessary (Gaytan, 2009). The shortage of acetonitrile imposed limitations on the analysis of 1,4-DMN in this study, in addition to a number of other analytical methods in different fields. Therefore, developing another effective method for the extraction and HPLC analysis of 1,4-DMN using an alternative solvent was required. In RP-HPLC, the UV cut off wavelength is an important factor for solvent selection and should be lower than the absorbance λ_{max} for the target analytes in order to avoid high background absorbance. Methanol was considered for use as an alternative to ACN due to its wavelength cut off (205 nm), polarity and good solvent properties. A new isocratic reversed phase HPLC-UV method was successfully developed for the analysis of 1,4-DMN and its internal standard 2-MeN using 90% methanol as the eluant with high resolution, precision, linearity and LOD/LOQ. This HPLC method is suitable to apply to extracts obtained in the quantitative

determination of 1,4-DMN residues in potato samples and other environmental samples. Oteef (2008) developed a method for the routine analysis of 1,4-DMN residues in potatoes using an extraction solution mixture of acetonitrile: 2-propanol 7:3 (v/v) containing the internal standard 2-methylnaphthalene at 50 °C for 15 min with occasional swirling. The extraction solution (ACN/PROP) used in this method was found to provide good extraction efficiency and to be compatible with the mobile phase 70% ACN. 1,4-DMN in extracts of potato peel at a low level of 0.005 µg/mL was successfully separated and quantified with satisfactory precision (RSD% of 8.6). In the present study, the method developed for the separation of 1,4-DMN would need to be tested for extraction compatibility with these solvents before using for extracts obtained from potato samples. The same arguments can be followed for the analysis of CIPC as well.

In reviewing the literature, no validated analytical method was found specifically for the combined analysis of both CIPC and its degradation product 3-CA by HPLC-UV. Unpublished work conducted by researchers at the University of Glasgow, developed an analytical method for the extraction and HPLC analysis of CIPC using ACN as a solvent, but did not include the analysis of 3-CA. During the ACN global shortage, it was deemed worthwhile to develop an analytical method for the extraction and quantitative analysis of these compounds using IPC as the internal standard and methanol as the solvent for the both extraction and for the eluant. Using 62% MeOH as the mobile phase provided good separation of all three compounds, 3-CA, IPC and CIPC, with a short run time (15 minutes). A short run time is usually required to analyse more samples on a daily basis. However, one of the problems that were faced during the development of this method was the appearance of an impurity peak (this may have been caused by an impurity in the methanol produced during manufacture or another unknown source). The retention time of this peak was close to the retention time of the 3-CA peak causing overlapping of peaks and this affected the accuracy of the quantitative determination of 3-CA particularly at very low concentration (≤ 0.02 µg/mL).

Most often, impurity peaks can be eliminated by maintenance of the HPLC apparatus, control of the mobile phase composition and avoiding contamination of the sample. Otherwise, identification and control of these peaks may become very complicated (Yang et al., 2010). To overcome the overlapping peaks and achieve high resolution of 3-CA particularly at low concentration (~ 0.02 µg/mL), the mobile phase was reduced to 60%, but this increased the run time from 15 to 20 minutes. Reducing the mobile phase to 55%, with a shorter run time of less than 10 minutes was possible for the analysis of 3-CA, only

when there was no CIPC present. This method was validated in terms of precision, linearity and limit of detection and quantification and is suitable for the quantitative determination of CIPC and 3-CA in potatoes and environmental samples.

Following development of these chromatographic methods, they were considered suitable to apply to the analysis of the studied compounds in both potato wash water and in commercial potato samples.

Pesticide residues in food are affected by storage, handling and processing (Gonzalez-Rodriguez et al., 2011). Washing with water is an important stage during processing and is essential to reduce the residue levels prior to human consumption and commercial use. One of the most significant current discussions in the potato industry regarding the application of potato sprout inhibitors is the concern about the residues of these compounds in wash water effluent and their fate in the environment. Washing treated potatoes during pre-packing or processing, releases chemical residues and associated sediment to washing water, which may be removed to landfill or discharged into watercourses without receiving any treatment (Park, 2004). Additionally, there is environmental interest in the degradation products of pesticides (e.g. 3-CA) because their concentration is continuously increasing in water and soil due to their low degradation (Angioi et al., 2005). In this case, concern should be rising particularly if the residues present in watercourses are at a high level.

In order to produce a reliable determination of pesticides in wash water samples specific details are required to evaluate the performance of the analytical procedure. Prior to quantitative analysis of 1,4-DMN, CIPC and 3-CA using 2-MeN and IPC as internal standards, in real water samples, it was necessary to assess the influence of laboratory conditions on the accuracy of measurements. The solubility of these compounds in water was assessed. Because 1,4-DMN and 2-MeN are polycyclic aromatic hydrocarbons, they have low water solubility (11.4 and 24.6 mg/L respectively). To prepare aqueous solutions of these compounds, an organic solvent should first be used to dissolve an exact weight of these materials with which to prepare stock solutions. Then, an aqueous solution can be prepared from the organic stock solution (Wolska, 2008). Aqueous standards of 1,4-DMN were prepared from a stock solution in acetonitrile by continuous stirring with a magnetic stirrer for 24 hours to ensure complete dissolution of 1,4-DMN. Following this same procedure to prepare water standards, no big difference was found compared to standards prepared in acetonitrile. Because CIPC and 3-CA are more soluble in water, standard solutions of these compounds were prepared by directly dissolving them in water. These

aqueous standards of CIPC and 3-CA were compared with those prepared in methanol. This showed no significant difference for 3-CA and a very small statistically significant difference for CIPC. Because of the low water solubility of CIPC (89 mg/L) compared to 3-CA (5400 mg/L), it requires great care in accurately weighing the small amount of CIPC needed to prepare a stock solution in water.

For the accurate determination of pesticides in water samples, all the steps undertaken prior to the final measurement including sampling, sample preparation and extraction procedures should not affect the actual amount of the pesticide present in the sample. The sources of the inaccurate quantitative measurements are varied. Adsorption of pesticides is an important consideration during the determination of acute lethal, chronic toxicity and residue accumulation in aqueous systems in addition to their persistence studies in water. Adsorption of pesticides should be considered when their water solubility is less than 1 $\mu\text{g/L}$ (Sharom and Solomon, 1982). Adsorption onto the walls of glassware and other devices used for sampling, transport and isolation are a major process causing imprecise determination of PAHs in water samples (Wolska, 2008). Typically, aqueous solutions of poly aromatic hydrocarbons have very low solubility ranging from mg/L to $\mu\text{g/L}$, which again can lead to adsorption problems. In this study, 1,4-DMN and 2-MeN showed no adsorption onto new volumetric flasks but a small adsorption onto old glass containers. However, using plastic containers and filters resulted in a strong adsorption of these compounds. In contrast, studying the potential adsorption of CIPC and 3-CA onto glass and plastic laboratory ware from aqueous solution showed no adsorption of 3-CA and good recoveries for CIPC with most of these materials. In conclusion, quantitative analysis of 1,4-DMN, CIPC and 3-CA in water samples is possible using the selected laboratory ware, the adsorption of 1,4-DMN onto the filters can be avoided by using centrifugation.

Pesticide residue measurements are required to establish maximum residue limits (MRLs) and subsequently for enforcement purposes and for dietary intake assessment. The MRL can include pesticide metabolites and photolysis products which have similar toxicity properties to the parent substance (Gonzalez-Rodriguez et al., 2011). In 2009, a document (SANCO-4967-2009-rev-3) relating to European Communities Commission regulations set out the foods to be sampled and the product/pesticide combinations to be tested during the years 2010, 2011 and 2012. The text related to chlorpropham stated that chlorpropham and 3-chloroaniline should be combined and expressed as chlorpropham. It was recommended that the MRL for CIPC was to be 10 mg/kg in potato samples and this should include its metabolite 3-CA (European-Commission, 2009). Therefore, in order to assess the residues

of CIPC and 3-CA, it was necessary to develop suitable, precise and rapid analytical methods permitting good extraction and interference free quantification that can be applied to large numbers of samples daily. The routine method used at the University of Glasgow for the determination of CIPC in potato samples is based on Soxhlet extraction using hexane as the solvent, rotary evaporation and GC-FID detection. Another simpler method based on a soaking extraction using acetonitrile as the solvent coupled with HPLC-UV analysis is also used. These two methods are not validated for the determination of 3-CA. As the HPLC-UV method provides greater sensitivity than GC-FID analysis, without the need for extract concentration and further clean up steps, this method was investigated for residue determination of both CIPC and 3-CA using an internal standard of IPC. In addition, to overcome the problem of the acetonitrile shortage, methanol was tested as a replacement for ACN as both the extractant solvent and the eluent as discussed earlier.

CIPC as an organochlorine pesticide is non-systemic (Stanciu et al., 2008). It can not penetrate into the potato tuber and mainly remains on the peel surface, its potential absorption depends on the formulations, lipophilicity and the active ingredients. Therefore, for the extraction of CIPC residue, the potato peel can be taken to represent the residue in the whole potato tuber.

A new methanol-soaking-HPLC analytical method was developed through overnight (~ 16 hours) soaking of chopped potato peel (5 g) in methanol (20 mL) used as the extracting solution containing IPC as the internal standard. The extract was filtered and finally analysed by HPLC. This method was validated in terms of the limit of quantification giving values of 0.01, 0.05 and 0.02 mg/kg in the whole tuber for CIPC, IPC and 3-CA respectively (using organic potato peel extract). The non-appearance of CIPC and related compounds in the extract of organic potatoes was the reason for selecting organic potatoes for the purpose of this study. The presence of CIPC and related compounds and co-extractives from the sample can affect the chromatographic analysis in significant ways causing difficulty in the identification and quantitative determination of the studied compounds.

The accuracy of the new method was measured through a recovery study by spiking organic potato peel. This gave high values of up to 90% for both CIPC and IPC at three spiking levels 0.8, 8.0 and 80 µg/g. 3-CA showed unexpected results of very poor recovery (< 23%). In particular, no peak was detected at the lowest level (0.8 µg/g) of spike. This low recovery of 3-CA was for a peel spiking time of just one hour. Increasing the contact

time between the analyte and the matrix may result in a much lower recovery of the analyte. The recovery of an analyte from spiking the matrix under laboratory conditions for known times is unlike real samples under commercial store conditions. In spiked samples, the analyte may well not reach equilibrium whereas the analyte in commercial samples may do, taking into account the long time between application and analysis. Thus, the recovery from treated potato samples is expected to be lower than that from spiking organic peel samples.

The methanol-soaking-HPLC method was compared with Soxhlet-GC which is a standard method for residue determination of CIPC in commercial potato samples within the University of Glasgow laboratories. The new method provided higher efficiency through the soaking extraction procedure (23% higher) than with Soxhlet extraction. There are many differences between the two extraction methods such as: the weight of the peel sample, extraction procedure, extraction time and extracting solvent. Soxhlet extraction involves many steps that may be a major source of the reason that results in the lower extraction residue. Analysing the same extracts derived from the Soxhlet extraction showed that the HPLC chromatographic technique provided higher values (13% higher) than that for GC analysis. This can be interpreted as different sources of CIPC loss during preparation of the extract for GC analysis including: the rotary evaporation, volatilisation, transfer of the extract and inadequate rinsing of the extract flask with the solvent (incomplete quantitative transfer).

The advantages of the methanol-soaking-HPLC method were as follows: the small volume of methanol solvent required, reduced number of steps in sample preparation and extraction and the analysis of a larger number of potato samples daily (~ 20). However, this method showed poor recovery of 3-CA. Analysis of treated potato samples from commercial stores by application of this method showed high residues of CIPC, some of which exceeded the MRL. High residues of 3-CA were also found and importantly, this was in spite of the low recovery of the method.

The low recovery of 3-CA was attributed to incomplete extraction and non-extractable bound residues within the potato peel matrix. It is well known that plants can incorporate pesticides and their metabolites into bound and non-extractable residues. These residues resist solubilisation in common solvents and are therefore not accessible to standard residue analysis (Sandermann, 2004). The non-extraction of the chemical residue from the sample matrix depends on its chemical properties and reactive functional groups, time

course of binding, environmental factors influencing binding rates, binding sites and mechanisms and the extraction procedure (Skidmore et al., 2002; Roberts, 1984). Aniline compounds and their derivatives have a high potential adsorption and form significant amounts of non-extractable residues in plants (Roberts, 1984). The understanding of this binding process and non-extractable residues is not clear due to the complex structure of the plant matrix.

To improve the extractability of 3-CA from the potato peel, many attempts were conducted testing different potato varieties, solvents, extraction methods, spiking times and different parts of the potato tuber. Four mechanisms were suggested for the low recovery including volatilisation, reaction with potato components, enzymatic activity and ion exchange binding related to pH. The possible loss of 3-CA due to volatilisation during the spiking period was proved to be unlikely to occur under the experimental conditions, despite the high vapour pressure of this compound. 3-CA as an aromatic amine contains an amino group which may cause high binding with potato matrix molecules. Pesticides are incorporated into plant tissues through proteins, lignins, pectins, hemicelluloses and cutins by covalent or non-covalent bonds (Sandermann, 2004). Pectins contain ester groups which can react with the nitrogen amino group of chloroaniline by nucleophilic substitution. It was reported that 3-CA can be copolymerised into the lignin, the hypothesised mechanism was an addition of this compound to a quinone methide intermediate (Roberts, 1984).

In the present study, the recovery of 3-CA was decreased when spiking glucose in the presence of water; this suggested that a Schiff base reaction or hydrogen bonding might be occurring between the carbonyl group of glucose and the amino group of 3-CA. However, direct contact between aqueous solutions of 3-CA with other solutions of possible chemicals present in potato showed no loss of 3-CA.

Another possible explanation for the poor extraction of 3-CA was suggested to be enzymatic activity of the polyphenolase enzyme either by direct breakdown and oxidation of 3-CA or by a Schiff base reaction of the amino group of the latter with the quinone products of PPO enzyme activity. Treating potato juice with antioxidants (ascorbic acid and sodium dithionite) or heating to reduce the activity of the PPO enzyme prior to spiking with 3-CA, showed excellent extraction recovery compared with spiking untreated juice. This may suggest a considerable role of enzymatic oxidation in the poor extractability of 3-CA. Enzymatic inhibitors, either as antioxidants or used to lower pH

showed an improvement in the extraction of 3-CA from potato peel also. However, the higher acidity of sulphuric acid seemed to have considerably effect in enhancing the extraction of 3-CA from potato peel relative to antioxidants.

This role of acidity cannot be interpreted to ion exchange, as the pK_a of 3-chloroaniline is 3.52, meaning that this compound will be present as the nonionic species in the environment. The acidity seems to be a direct effect of the pH of the extracting solution of 3-CA. It was also observed in unreported work in this study that the lowest adsorption of 3-CA onto the potato peel in aqueous solution, occurred at low pH. Using the high acidity of sulphuric acid combined with methanol as the extracting solution for spiked peel improved the extraction recovery. Additionally, systematic solvent trials may be useful for various unextractable residues in plant (Sandermann, 2004). It was reported in one study of the non-covalent bound residue of chlorpyrifos-methyl on wheat grains, that the bound residue was not solubilised by water or methanol (Matthews, 1991). Optimisation of the composition of the methanol and water mixture found that 50% aqueous methanol solubilised 86% of the bound residue.

In the present study, optimising the extraction procedure and selecting 1 M H_2SO_4 in 50% methanol, at a temperature of 50 °C for a 24 hour extracting time achieved a recovery of up to 85%. No equilibration was reached at 24 hours, which means that higher recovery may be obtained using a longer extracting time. No breakdown of CIPC was occurred under these extraction conditions. This simple extraction method can be suitable for the determination of the residues of both 3-CA and CIPC from potato samples.

Applying the final method to commercial treated potato samples showed residue levels of CIPC lower than the MRL. The high residue of 3-CA detected might be attributed to thermal degradation during application, particularly as these potatoes received many treatments of CIPC from a solid formulation at 450 °C using metal pipes. Microbial degradation may also take place as these potatoes were stored for a long period of time. However, analysis of potato samples from two different stores (at between 8 and 10 °C) which had received the first application of CIPC at high (450 °C) and low (270 °C) temperatures showed no significant differences for both CIPC and 3-CA residues. A second application at a lower temperature showed a significant increase in the residue of both compared to the first application. This indicates a possible build up over time and with repeated application, microbial degradation of CIPC to 3-CA can also be expected due to the length of storage time.

Brajesh and Ezekiel (2010) found a correlation between the number of CIPC applications and the residue of CIPC remaining in potato tubers. The residue of CIPC was determined in tubers (using HPLC analysis) after first and second application of dust and aerosol treatments at storage temperatures 10 and 12 °C. The residue of CIPC in peel after the first dust application was high and declined during the storage period but increased after the second dust application. In this study, the residue of CIPC from the first aerosol application was 20 – 82% higher than from dust application with more effectiveness. There was a decrease in the residue during the storage period and then an increase in the residue level immediately after the second application of CIPC. The highest residue level determined in the peel was 20.17 mg/kg fresh weight, whereas very low concentrations of CIPC (ranging from 0.05 – 0.24 and 0.29 – 1.13 mg/kg respectively) were found in peeled and unpeeled whole tubers (Brajesh and Ezekiel, 2010).

It should be noted that some of the applied CIPC can be lost from the potato tuber due to volatilisation, but as found in this study this is not the case with 3-CA.

An implication of the findings in this study is that the presence of 3-CA in potato stores might be a big concern for the potato industry. The low recovery of 3-CA from potato tuber (< 10%) should be taken into account, particularly this was the recovery when the contact time is only one hour under laboratory conditions. Repeating the application of CIPC during long storage periods may lead in a much higher formation of 3-CA and high binding to the potato tuber and subsequently much lower extractability of this aromatic amine compound. Thus measurements of 3-CA by this and similar methods will be underestimated, especially at long storage times.

7.2 Recommendations for future work

Some investigations are recommended for further work including:

The chromatographic methods developed for the separation of 1,4-DMN will serve as a base for future studies to analyse the extract from potato and environmental samples or development extraction methods using methanol as the extracting solvent and it is compatible with the eluent.

The impurity peak was one problem found in this study when testing different batches of methanol. More investigation is required to identify this impurity peak. Mass spectrometry analysis may be one of the suggestions to determine what the solvent impurity is.

Information should be available on the residue of the studied compounds present in potato washing water during processing. Detecting the level of these chemicals that run off into watercourse is very important due to their potential risk in the environment and aquatic systems. The allowed residue level of these chemicals in watercourses should also be set out. Therefore, studies are required to assess first, the level of the residue of these compounds in wash water and the associated sediment, particularly as these residues may be adsorbed on to solid particles. Additionally, adsorption of these compounds on potato samples and sediment in aqueous solution should also be investigated.

The high cost of acetonitrile and adsorption of 1,4-DMN on plastic and filters were major obstacles in the research to determine the presence/concentration of this compound in water samples. Because this investigation is very important, further work is recommended. The glass materials are acceptable to use in future experiments and the slight loss of 1,4-DMN due to adsorption can be controlled by applying cleaning procedures using: Decon 90, 1 M NaOH, 1 M H₂SO₄ and ACN. However, plastic materials should be avoided. To find a means to filter the sample, possible alternative filters can be tested such as PTFE, nylon, cellulose nitrate, mixed cellulose esters and polycarbonate. Centrifuging using glass centrifuge tubes may be acceptable but attention should be given to ensure there is no plugging of the HPLC column. Using a very large volume of sample solution to saturate the adsorbed sites of the filter may also be practical.

The dietary risk of pesticides and their metabolites cannot be assessed if their residues are bound, thus raising issues regarding the toxicological significance of these bound residues (Skidmore et al., 2002). Binding of 3-CA to potato samples which could be serious for human consumption is the main problem identified and added to the knowledge by this study. The bound residue of 3-CA on potato samples has never been investigated before, so the actual amount and the mechanism behind this bound residue in these samples are still poorly understood hence further investigation is required. Radioactive labelling is one suggestion for understanding this binding mechanism in potato peel.

Degradation of CIPC to 3-CA during application is a major concern for the potato industry in the UK; hence improvement in its application is required. The effects of many factors on the residue of both CIPC and 3-CA in potato samples should be investigated further, including: fogger temperature, material of pipe surfaces, CIPC formulation, rate of CIPC application, number of applications, store conditions and storage time.

Additionally, the possible microbial degradation of CIPC to form 3-CA during storage is another issue that requires more investigation to be resolved.

To study the distribution of 3-CA in potato stores, samples from different places in the store should be taken and analysed. The distribution of 3-CA within potato tubers should also be investigated, analysing different layers and different sites within the potato tuber. In addition, the availability of 3-CA as a result of thermal degradation of CIPC during cooking or frying is a worthwhile issue and must be taken into account.

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Determination of the Potato Sprout Inhibitor Chlorpropham and its Metabolite 3-Chloroaniline in Potato Samples

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Abstract

A simplified method based on soaking overnight extraction coupled to HPLC - UV analysis was developed for the simultaneous determination of the residue levels of the potato sprout inhibitor chlorpropham (CIPC) and its metabolite 3-chloroaniline (3CA) in potato samples. The method gave values approximately 25% higher when compared with a standard Soxhlet -GC method. The results of spiking different layers from the potato tuber showed a high recovery of CIPC (> 95%) in all layers but the recovery of its metabolite 3CA was lower than 50% in the pith and 5% in both cortex and skin.

Introduction

Chlorpropham (CIPC) is the main pesticide used as a sprout suppressant in the UK to prolong the storage period and maintain the quality of stored potatoes. Degradation during CIPC application in the store by thermal fogging or later microbial breakdown of CIPC on the potato during storage can produce 3-Chloroaniline (3CA) (Nagayama and Kikugawa, 1992; Worobey and Sun, 1987). For human risk assessment, there is a concern over 3CA which has a formula similar to a well-known carcinogenic compound 4-chloroaniline. Moreover, 3CA is recognized to be a toxic water pollutant and harmful to aquatic life according to European Community pollutant Circular No 90-55 (1990). From 2007, the maximum residue limit (MRL) for potatoes treated by CIPC is fixed at 10 mg/kg for human consumption. Recently the European Communities Commission recommended that both 3CA and CIPC are included in the maximum residue level value from 2011 (SANCO, 2009). Therefore, determination the level of 3CA in potatoes is very important for the potato processing industry. The main objective of this work was to develop and validate an analytical method to extract and analyse both CIPC and its metabolite 3CA residues in stored potatoes tubers that have been treated with CIPC.

Material and Methods

Analytical grade reagents were used in this study: Chlorpropham (purity 95%) was supplied by Sigma, 3-chloroaniline (99%) was obtained from Aldrich, and propham from Riedel- de Haën (Sigma-Aldrich). Methanol and Hexane that used were HPLC grade.

The HPLC system comprised a GILSON® 234-auto sampler, Cecil 1100 Series pump, Phenomenex® ODS-2 250 x 4.60 mm 5µ Sphereclone column, and Thermo Separation UV100 detector at 210 nm coupled with Dionex Peaknet software. An isocratic method was employed with 62% (v/v) methanol as mobile phase at a flow rate of 1.5 ml/m, 20µl sample injection volume, and chromatographic run time 15 minutes. GC analysis was performed on a Hewlett Packard HP 5890A coupled to a Flame Ionization Detector (FID) with HP 7633A auto sampler unit and DB-1 column (30 m, 0.53 mm i.d., 1.5 µm film thickness).

The procedure of soaking extraction method involved peeling the potato, chopping the peel into fine pieces and mixing to obtain a homogenous sample. A 5g peel sample was weighed into a 100 ml screw top jar, then 40 ml methanol containing the internal standard 10 µg/ml Propham (IPC) was added as extracting solution and left to soak overnight (~ 18 h) at room temperature. Next day, the extract was filtered and transferred into HPLC vials through syringe (2 ml) and 0.2µm PTFE membrane syringe filter.

The soaking – HPLC method was validated and compared with Soxhlet extraction which is the standard method at University of Glasgow. This standard method was performed on the remainder of the peel for each tuber which was placed into a Soxhlet thimble that contained 10 g sodium sulphate then extracted with 150 ml of hexane for 2 hours. The extract was then concentrated to 1 ml using a rotary evaporator, and 200 µl of 1000 µg/ml Propham (IPC) added and the volume was made up to 2 ml for GC analysis.

Determination of pesticide residue and its metabolite in potatoes samples

The soaking-HPLC method was applied to determine the residues of the parent pesticide and its metabolite. Randomly, 30 potatoes tubers were selected from the bags obtained from UK processing stores that had received CIPC application.

Spiking organic potato with the pesticide and its metabolite

In order to compare the recovery of CIPC and 3CA from the various layers of the potato tuber: skin, cortex and pith. 2.5g of each layer of the organic potato tuber was spiked with 200µl of a mixture of 100µg/ml CIPC and 3CA and left for 1 hour, then 20 ml methanol containing 1µg/ml IPC was added prior to extraction by overnight soaking.

Results and Discussion

A robust method based on reversed phase HPLC with UV detection coupled with soaking overnight extraction was developed for the separation and determination of CIPC and 3CA in potatoes extracts. Applying optimum chromatographic conditions achieved a best separation of chlorpropham, propham, and 3-chloroaniline at the retention time (~ 12, ~ 6, and ~ 4 minutes respectively).

The limits of detection (LOD) and quantification (LOQ) for the soaking- HPLC method were determined by ten replicate injections (n=10) of a 0.05 µg/ml mixture of CIPC, IPC and 3CA prepared in an extract of organic potato. LOD and LOQ of CIPC, IPC and 3CA reported low values (0.002, 0.015, and 0.002) (0.008, 0.051 and 0.005) mg/kg respectively. To validate the soaking-HPLC method, it was compared with a standard Soxhlet – GC method as shown in Figure 1. The regression line shows good correlation between the CIPC residues in potato tubers analysed by both methods, however, the soaking – HPLC method gave results approximately 25% higher than Soxhlet – GC standard method. This difference can be attributed to the time of extraction and the higher polarity of the methanol compared to hexane.

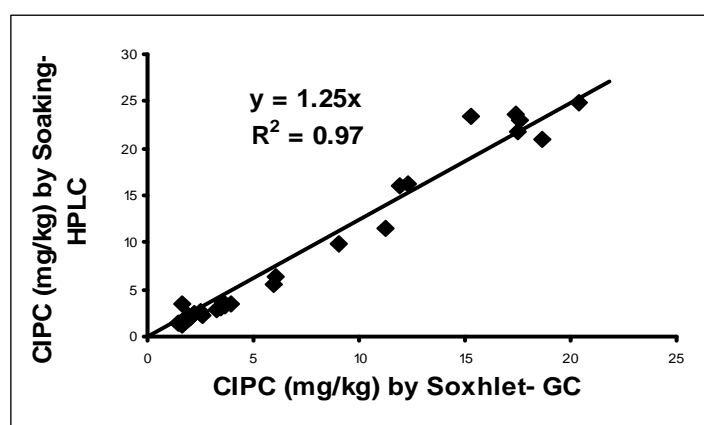


Figure 1: Shows the correlation between CIPC extract by methanol soaking extraction- HPLC analysis and hexane Soxhlet extraction- GC analysis.

Determination of pesticide residue and its metabolite in potatoes samples

The developed method is easy to use, efficient and inexpensive, therefore it was applied to determine the residue levels of the parent pesticide chlorpropham and its metabolite 3-CA in treated potatoes. The results of residue levels in 30 individual potatoes were in the range (1.16-24.79) and (0.06-0.34) of CIPC and 3CA respectively, although, 3-CA was not detected in some tubers. From the residue results, some samples of potatoes exceeded the MRL level of CIPC but they may have been treated recently. This variability of residue concentrations of CIPC and 3CA can be attributed to various factors related to the storage conditions, storage time, potato location in the store, circumstances of CIPC application into the store, peel sample preparation and the extraction process (Park *et al.*, 2009).

The recovery of CIPC and 3CA from spiking different layers from potato tuber

The recovery efficiency of soaking–HPLC method for CIPC and 3CA from spiking different layers of the potato tuber produced high recovery of CIPC (> 95%) in all layers but the recovery of its metabolite 3CA was lower than 50% in the pith and 5% in both cortex and skin as shown in Figure 2.

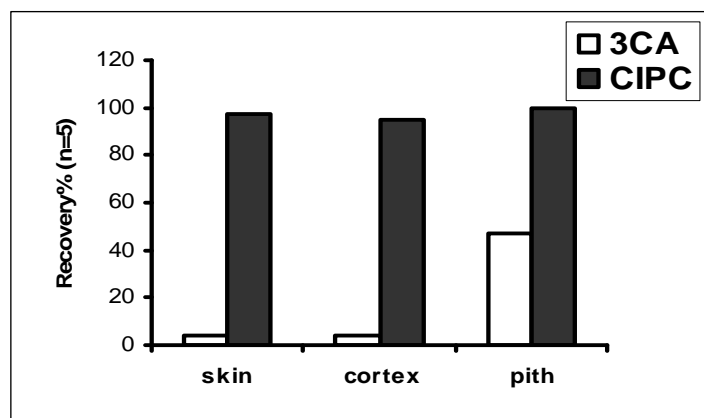


Figure 2: Shows the recovery of CIPC and 3CA from spiking different layers of potato tuber.

The low recovery of 3CA could be due to binding or instability of 3CA with the potato tissues. Moreover, to the changes in the structural tissues and biological materials for these various layers within the potato tuber tissues that could lead to difficult extraction of 3CA as explained by others (JMPR, 2001; Still *et al.*, 1981; Worobey *et al.*, 1987). From this poor recovery of 3CA found particularly from spiking potato skin and cortex which less than 5% recovery, it can be concluded, the residue concentration of 3CA represents approximately 5% of the actual amount present in the potato tuber treated with CIPC, and this low recovery is due to incomplete extraction. Therefore, further work will be required to find a suitable way to improve the extraction of 3-CA from the potato tuber to obtain a higher recovery and investigate possibly losses of 3-CA from spiked potatoes.

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Extraction of residues of chlorpropham and its metabolite 3-chloroaniline in treated potatoes using various methods

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ABSTRACT

In the UK, the potato industry needs to store large quantities of potatoes. The major concern is sprouting during storage, which causes for deterioration in quality. Chlorpropham (CIPC) is the main sprout suppressant chemical used and is applied as a hot fog to the stored potatoes. However, residues of CIPC and its main breakdown product 3-chloroaniline (3CA) can be found on the treated potatoes. For human risk assessment, there is concern over 3CA. Recently, the European Communities Commission set a maximum residue level value of both 3CA and CIPC to come into force from 2011. Therefore, a method is required to extract and estimate the residues of both Chlorpropham and 3-Chloroaniline in potato samples.

Methods for analysing CIPC are well documented but not for 3CA. In our previous work, a simplified method was developed and validated to extract and analyse CIPC based on soaking overnight in methanol coupled to HPLC - UV analysis using 62% methanol as eluent. When applied to potato skin spiked with CIPC and 3CA the results showed a high recovery of CIPC (> 95%) but the recovery of its metabolite 3CA was less than 5%. The reason of this poor extraction was thought to be the binding of 3CA to potato skin due to a positive charge on the 3CA molecule. To overcome this, potatoes were extracted by soaking in a 50:50 mixture of methanol and 1M H₂SO₄ for 24 h. Experimental recoveries (n=5) at a spiking level of 8 mg kg⁻¹ were above 60% and 80% at ambient temperature and 50°C respectively.

The final part of this study was to look at potato samples taken from UK stores at the end of the 2009–2010 storage season. The potato skin was extracted applying three different methanol-extracting solutions for 24 h (100% methanol at lab temperature, 50% methanol in 1 M H₂SO₄ at lab temperature and at 50°C). The results showed high residue levels of 3CA using acidified extraction at 50°C while the residue results for CIPC were unaffected. No samples exceeded the MRL level for CIPC (10 mg kg⁻¹). Further studies at the start of the current storage season are comparing the formation of 3CA using high and low temperature fogging at two temperatures (450°C and 270°C) and application rates of 14 and 12 g tonne⁻¹ respectively.

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